Chocolate polyphenols protect against reactive nitrogen species via a novel mechanism of nitrosation : biological implications

Lee, Stephanie Ho Yan

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A THESIS SUBMITTED BY

STEPHANIE HO YAN LEE

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD) IN BIOCHEMISTRY

FROM THE UNIVERSITY OF LONDON

ANTIOXIDANT RESEARCH GROUP

WOLFSON CENTER FOR AGE-RELATED DISEASES

KING'S COLLEGE

UNIVERSITY OF LONDON

ABSTRACT

There is considerable interest in the health benefits of dietary flavonoids due to their abilities to scavenge reactive oxygen and nitrogen species and their potential beneficial role in gene expression. Initial studies described in this thesis demonstrate that the mechanism by which the catechin and epicatechin flavanols scavenge reactive nitrogen species (RNS) formed from acidic nitrite and inhibit tyrosine nitration is through the formation of nitrosated products, as confirmed by LC-MS/MS.

On the basis of this finding, studies were designed to examine the bioavailability, cytotoxicity and the biological activity of nitrosated catechins (NCs). The absorption and metabolism of NCs relative to catechin and other flavonoid molecules were first investigated using an *ex vivo* isolated rat jejunum model. While catechin was absorbed and metabolised as glucuronidated and methylated conjugates, no absorption or metabolism of NCs was detected in the serosal fluid. Similar results were obtained when the apparent permeability (P_{app}) and metabolism of NCs was compared with other flavonoids using a human Caco-2 cell model. Catechin was mainly absorbed as conjugates and metabolites whereas little absorption of NCs was detected. The determination of the P_{app}s in relation to other flavonoids revealed that in general there is an increase with enhanced lipophilicity. Further studies were undertaken to investigate whether nitrosated catechins elicit cytotoxic effects. No toxicity was observed in either Caco-2 cells or the human fibroblasts systems. In addition, NCs were demonstrated to protect fibroblasts from oxidative stress induced cell death in a concentration-dependent manner. These findings demonstrate that, contrary to the mechanism whereby flavonoids protect against peroxynitrite-induced stress, via nitration or oxidation of the flavonoid moiety, catechin protect against RNS derived from acidic nitrite as in the gastric lumen through the novel mechanism of flavonoid nitrosation. Furthermore, NCs are not absorbed by the gastrointestinal tract and are not cytotoxic.

DECLARATION

DEDICATION

To My Parents and Godfather

ACKNOWLEDGEMENT

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CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
A to B	Apical to basolateral permeability
B to A	Basolateral to apical permeability
BSA	Bovine serum albumin
CA	Catechin
cLog P	Calculated lipophilicity (octanol/water partition)
CYP	Cytochrome P_{450}
DAD	Photodiode array detection
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPPH$^{\bullet}$	2,2-diphenyl-1-picrylhydrazyl
EC	Epicatechin
ECG	Epicatechin gallate
EDR	Endothelium-dependent relaxation
EDTA	Ethylene diamine tetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
FEK4	Normal human dermal fibroblasts derived from foreskin
GI tract	Gastrointestinal tract
GLUTs	Facilitative glucose transporters
H_2O_2	Hydrogen peroxide
HBM	HEPES-buffered incubation medium
HBSS	Hanks' balanced salt solutions
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
IgG	Immunoglobulin G
IL	Interleukin
JNK	c-Jun N-terminal kinase
LC-MS	Liquid chromatography-mass spectrometry
Log P	Octanol/water partition
MAPK	Mitogen-activated protein kinase
MDCK-MDR1	Madin-Darby canine kidney type II cells transfected with the full-length cDNA for human multidrug resistance gene 1
Me-CA	Methylated catechin
Me-EC	Methylated epicatechin

MEM	Minimum essential medium
MRP1	Multidrug resistance protein 1
MRP2	Multidrug resistance protein 2
MTT	3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide
4MU	4-methylumbelliferone
4MUG	4-methylumbelliferone glucuronide
MW	Molecular weight
NC's	Nitrosated catechins
NC1	Nitrosated catechin 1
NC2	Nitrosated catechin 2
NC prep	Nitrosated catechin preparation
NHPA	3-nitro-4-hydroxyphenylacetic acid
NHPL	3-nitro-4-hydroxyphenyllactic acid
NMR	Nuclear magnetic resonance
NOS	Nitric oxide synthase
PAMPA	Parallel Artificial Membrane Permeability Assay
P_{app}	Apparent permeability
PBS	Phosphate-buffered saline
PDA	Photodiode array detection
P-gp	P-glycoprotein
PKC	Protein kinase C
PRPs	Proline-rich proteins
R123	Rhodamine 123
R_f value	Retention factor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Retention time
SEM	Standard error mean
SGLT1	Sodium-dependent glucose co-transporter
SPE	Solid-phase extraction
SRB	Sulforhodamine B
TEAC	Trolox equivalent antioxidant capacity
TEER	Transepithelial electrical resistance
TLC	Thin layer chromatography
UWL	Unstirred water layer

CHAPTER ONE

General Introduction

Introduction

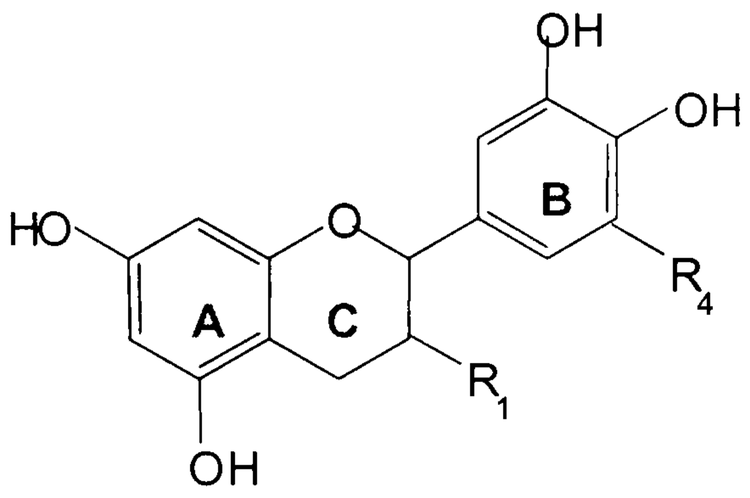
1.1 FLAVONOIDS

Flavonoids have become an intense focus of research interest because of their perceived protective role in diseases associated with oxidative stress. These include cardiovascular and neurodegenerative diseases, some forms of cancer and several other disorders such as diabetes and rheumatoid disease. Other biological effects of flavonoids have also been reported, including anti-allergenic (Shaheen *et al.,* 2001 #247, Benavente-Garcia 1997, Middleton 1998), anti-inflammatory (Middleton 1998, Harborne and Williams 2000, Mao *et al.,* 2002), anti-microbial (Shahat *et al.,* 2002, Olivero-Verbel and Pacheco-Londono 2002, Chung *et al.,* 1998), anti-ulcer (Saito 1998), immune modulating (Mao *et al.,* 2002, Sanbongi *et al.,* 1997, Mao *et al.,* 2000), enzyme modulating (Moini *et al.,* 2000, Romanczyk 1997, Cook 1996) and the ability to interact with cellular signalling pathways (Musonda and Chipman 1998, Tsai *et al.,* 1999, Gamet-Payrastre *et al.,* 1999, Schroeter *et al.,* 2001, Spencer *et al.,* 2001).

1.1.1 Major classes of flavonoids and their structures

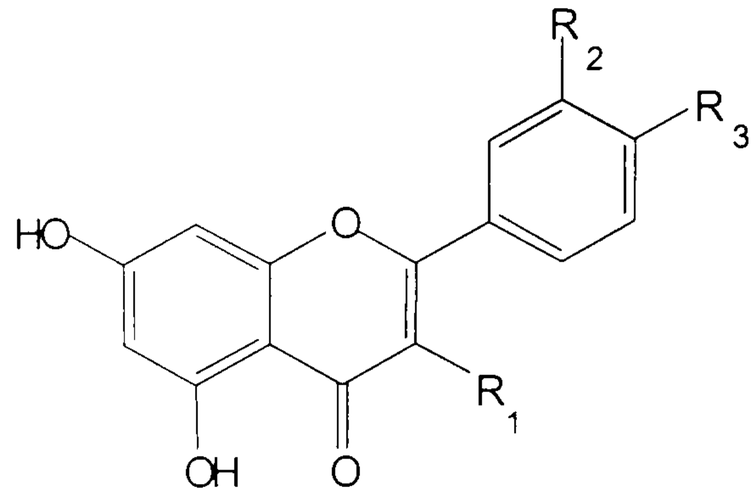
Flavonoids are ubiquitous in plants with more than 5000 naturally occurring forms identified so far (Harborne 1999). They are produced in the secondary metabolism of many plants and were considered as being non-nutritive to human for a long time. The major roles of flavonoids in plants include UV-B protection, antimicrobial, as well as protection against insect and animal consumption (Harborne and Williams 2000). Flavonoids are diverse in chemical structure and characteristics, with some being lipophilic occurring in the wax or leaf surface buds, whereas others are water-soluble occurring within plant cell vacuoles. The general chemical structure of flavonoids includes an A ring that is formed from three malonyl-CoA units, a B ring that is formed from phenylalanine and a C ring (Fig 1.1). Most flavonoids possess at least one hydroxyl group and are generally classified into several classes according to the different oxidation pattern of their central C ring (Harborne 1999). They are usually attached to sugar as glycosides, thereby increasing their stability and solubility in the aqueous environment within the vacuoles. While glucose is the most common sugar residue, others such as galactose, rhamnose and xylose are also found (Rice-Evans *et al.,* 1996).

Dietary intake of flavonoids mainly consists of five major classes (Figure 1.1), namely the flavones/flavonols, flavan-3-ols and the related procyanidins, anthocyanidins, hydroxycinnamates and flavanones (Rice-Evans *et al.,* 2000).



Flavanols

	R1	R4
Catechin	OH	H
Epicatechin	OH	H
EGC	OH	OH
ECG	gallate	H
EGCG	gallate	OH

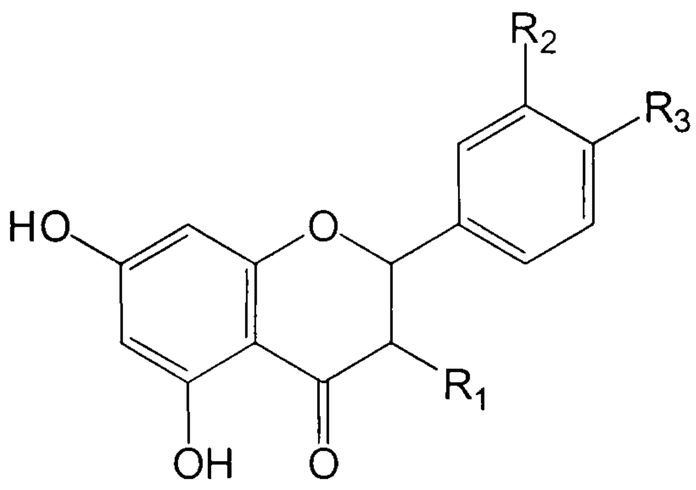


Flavonols

	R1	R2	R3
Quercetin	OH	OH	OH
Kaempferol	OH	H	OH

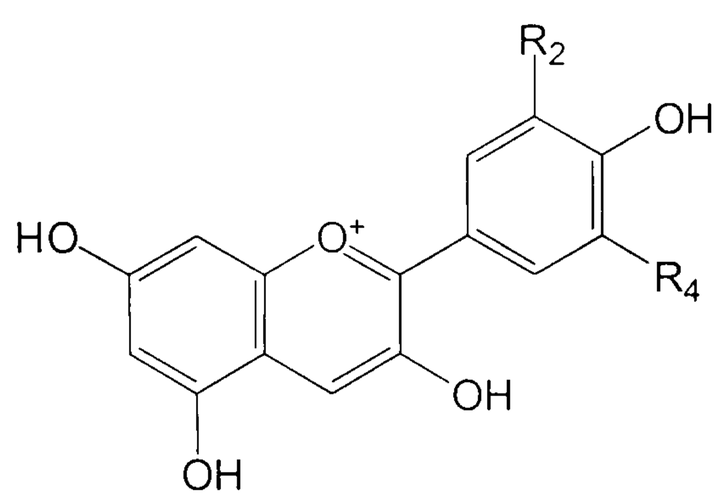
Flavones

Luteolin	H	OH	OH
Apigenin	H	H	OH



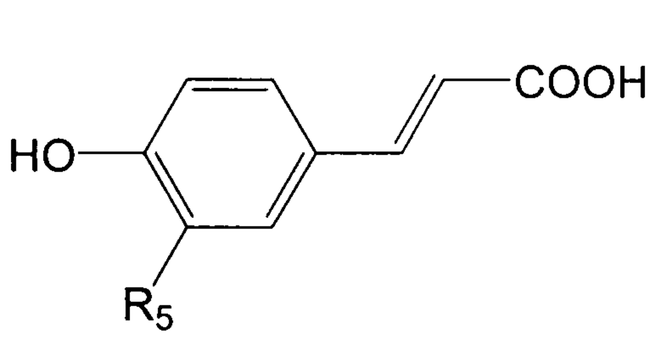
Flavanones

	R1	R2	R3
Taxifolin	H	OH	OH
Naringenin	H	H	OH
Hesperetin	H	OH	OCH_3



Anthocyanidins

	R2	R4
Cyanidin	OH	H
Malvidin	OCH_3	OCH_3
Delphinidin	OH	OH



Hydroxycinnamates

	R5
p-coumaric acid	H
Caffeic acid	OH
Ferulic acid	OCH_3

Fig 1.1 Structures of the five main classes of flavonoids. The major differences between the individual groups reside in the hydroxylation pattern of the ring structure, the degree of saturation of the C-ring, and the substitution in the 3-position.

1.1.1.1 Biological properties of flavonoids

The major biological property of flavonoids that has been studied extensively, is their ability to act as antioxidants. An antioxidant is defined as a compound that can significantly delay or inhibit oxidation of an oxidisable substrate, when present at a concentration lower than the substrate. On top of this, the resulting free radical formed must be a stable species that is incapable of bringing about further reaction. Flavonoids have been shown to act as scavengers of various oxidising species i.e. superoxide anion (Yuting *et al.,* 1990), hydroxyl radical (Jovanovic 1994, Jung *et al.,* 2003) or peroxyl radicals *in vitro* (Nakao *et al.,* 1998, Sawa *et al.,* 1999). Their abilities to scavenge reactive nitrogen species (RNS) such as peroxynitrite (Pannala *et al.,* 1997, Pannala *et al.,* 1998, Arteel and Sies 1999, Arteel *et al.,* 2000) and nitric oxide (Haenen and Bast 1999) have also been demonstrated.

Owing to the polyphenolic structures of flavonoids, they can function as antioxidants *in vitro* by virtue of their hydrogen-donating properties, and thus preventing oxidation of the oxidisable substrate (Rice-Evans *et al.,* 1996, Sichel *et al.,* 1991, Salah *et al.,* 1995). These antioxidant properties depend on the structural arrangements of the flavonoids' hydroxyl groups, which are summarised below:

- The *ortho* 3′,4′-dihydroxy structure in the B ring i.e. the catechol structure. This confers higher stability to the radical form, and participates in electron delocalisation.
- The 2,3-double bond with the 3-hydroxy group and the 4-keto group in the C ring for electron delocalisation from the B ring. This is an essential feature for the production of a stable radical, with quercetin being an example exhibiting this structure.
- The 3- and 5-OH groups with the 4-keto group in A and C rings, for maximum scavenging potential.

It has been suggested that the transition metal-chelating properties of flavonoids also contribute to their antioxidant properties (Paganga 1996, Mira *et al.,* 2002). The *o*-3′,4′-dihydroxy structure in the B ring and the 4-keto, 3-hydroxy or 4-keto and 5-hydroxy structure in the C ring are important structure for transition metal-chelation e.g. quercetin (Rice-Evans *et al.,* 1996, Rice-Evans 1997).

1.1.2 Dietary intake of flavonoids

Many recent studies have suggested that the consumption of flavonoids is associated with beneficial health effects. For instance, high flavonoid intake has been linked to a lower mortality from coronary heart disease and stroke in elderly men (the Zutphen elderly study) (Hertog *et al.,* 1993, Keli *et al.,* 1996). In addition, an inverse relationship between consumption of catechin and ischemic heart disease mortality has also been found (Arts *et al.,* 2001). Flavonoids are widely distributed in plants and the average daily intake of flavonoids by humans in Northern Europe has been estimated at around 50 to 150 mg per day (Hollman and Katan 1999). However, a much higher figure was obtained in an earlier study with an estimate of the daily dietary intake of polyphenols of approximately 1g, with flavonoids accounting for two thirds of the total (Kuhnau 1976). The difficulty and discrepancy in estimating flavonoid intake is mainly due to their structural diversity, lack of standardised analytical methods and variation of content in a particular foodstuff (Scalbert and Williamson 2000). Table 1.1 summarises the dietary sources of some commonly consumed flavonoids.

1.1.3 Absorption and metabolism of flavonoids

The biological properties of flavonoids *in vivo* are dependent on the structural changes as a result of their absorption and metabolism post-consumption. The human body is exposed to a wide array of foreign compounds, therefore a complex enzymatic mechanism has been developed to metabolise as well as to detoxify these substances. In general, all of these reactions can be assigned to one of two major categories called phase I and phase II reactions. Phase I reactions (Table 1.2) usually convert the parent compound to a more polar metabolite by introducing or unmasking a functional group (-OH, -NH_2, -SH), thereby enhancing the excretion of the compound. Often these metabolites are inactive, though in some instances activity is only modified. Some metabolites undergo a subsequent reaction, phase II reactions, which usually involve conjugation of the phase I metabolites with endogenous substrates such as glucuronic acid, sulphate, amino acid, glutathione etc. The resulting metabolites are highly polar and are readily excreted. Table 1.2 summarises the typical reactions occur during phase I & II metabolism in human.

*Flavonoid**	*Dietary sources*	*References*
Flavanols		
(+)-Catechin	Green and black teas, chocolate, apples, grapes, red wine	1, 2, 3
(-)-Epicatechin	Green and black teas, chocolate, apples, grapes, red wine	1, 2, 3
(+)-Epigallocatechin	Green and black teas	4
Flavanone		
Hesperetin	Lemon, lime, mandarin, sweet orange	5
Naringenin	Grape fruits, bitter orange	5
Taxifolin	Citrus fruits	4
Flavonols		
Kaempferol	Endive, leek, broccoli, radish, grapefruit, black tea	4
Quercetin	Onion, chives, broccoli, cranberry, apple skin, berries, olive, tea, red wine, elderberry, cherry tomatoes	4, 6
Myricetin	Tea, cranberry, grapes, red wine	4, 7
Flavones		
Chrysin	Fruit skin, honey, propolis	4, 8
Apigenin	Celery, parsley	4
Anthocyanidins		
Malvidin	Red grapes, red wine	4
Cyanidin	Cherry, raspberry, strawberry, grapes	4
Hydroxycinnamates		
Ferulic acid	Wheat, corn, rice, tomatoes, spinach, cabbage, asparagus, coffee	4, 9
Caffeic acid	White grapes, white wine, olives, olive oil, spinach, cabbage, asparagus, coffee, blueberry	4, 9
Chlorogenic acid	Coffee, apple juice, artichoke, aubergine, peach, blueberry, apricot	4, 9
p-coumaric acid	White grapes, white wine, tomatoes, sugar beet fibre, cereal brans	4, 9

Table 1.1 Dietary sources of some commonly known flavonoids. *Apart from the flavanol family most flavonoids occur as glycosides. (Adapted from Rice-Evans *et al.,* 1996). References: [1] (Hammerstone *et al.,* 2000); [2] (Arts *et al.,* 2000a); [3] (Arts *et al.,* 2000b); [4] (Rice-Evans *et al.,* 1996); [5] (Tomas-Barberan 2000); [6] (Aherne and O'Brien 2002); [7] (Ong and Khoo 1997); [8] (Siess 1996); [9] (Clifford 1999)

Type of metabolism	*Type of reaction*	*Location*
Phase I	Oxidation, hydroxylation, dealkylation, demethylation, epoxidation, deamination, dehalogenation[1]	Endoplasmic reticulum (ER)
	Hydrolysis, hydration	Cytosol
Phase II	Methylation, sulphation, acetylation, glutathione conjugation	Cytosol
	Glucuronidation	ER
	Amino acid conjugation	Mitochondria

Table 1.2 Typical reactions of Phase I and Phase II metabolism.
[1] All the reactions are catalysed by cytochrome P_{450}.

Although the liver is the major site of detoxification, the gastrointestinal (GI) tract plays an important role by providing a physical barrier to exogenous compounds, as well as being the second major site for detoxification. Major metabolism of exogenous compounds in the GI tract includes methylation and conjugation with glucuronic acid. Figure 1.2 describes the various sites of metabolism in the human body.

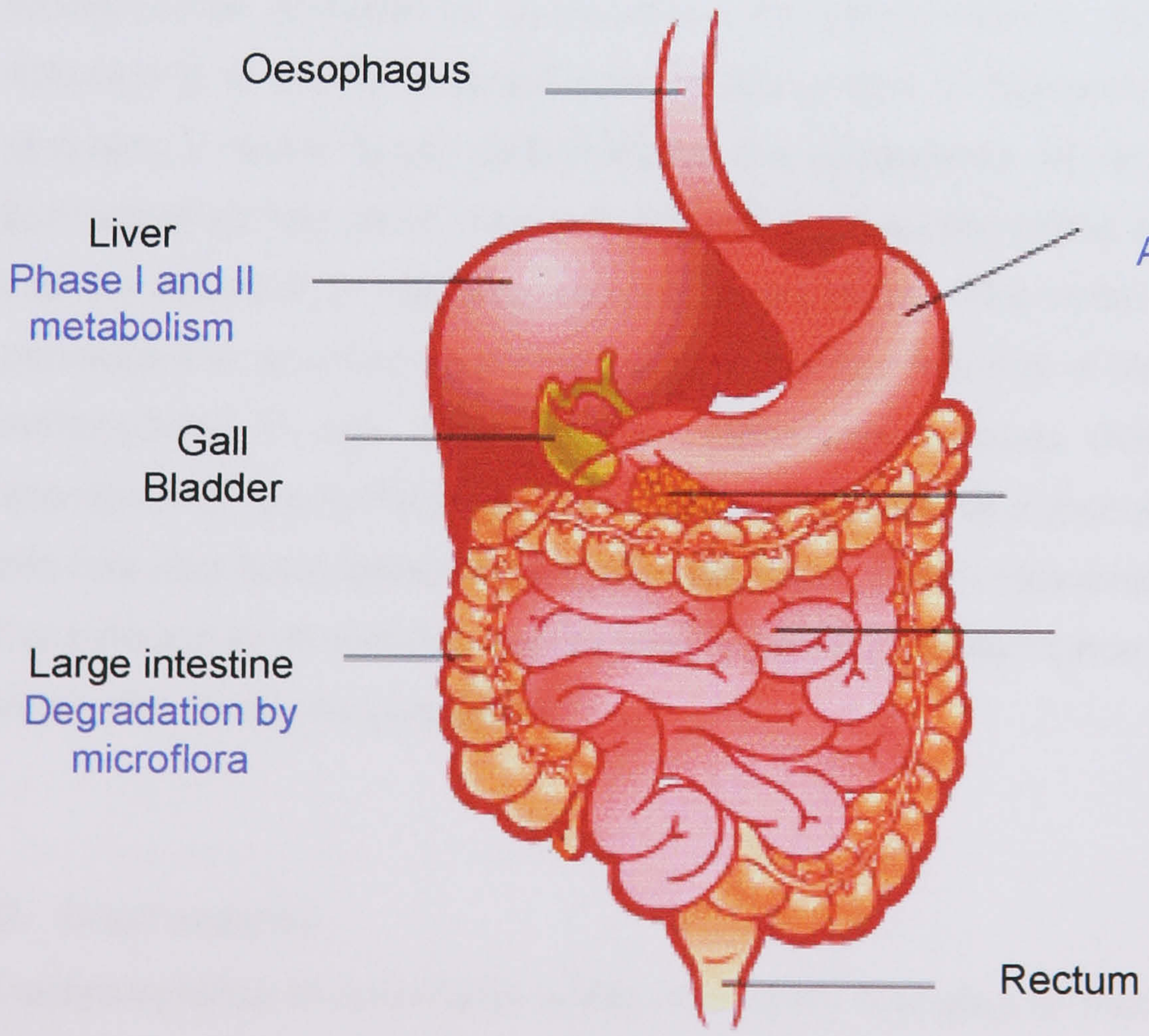


Fig 1.2 Metabolism of exogenous compounds by the human body.

A number of early studies hypothesised that flavonoids would not enter the circulation, either as the natural glycosides or as the hydrolysis product, the aglycone. Furthermore, cleavage of the β-glycosidic link only occurs in the colon by enzymes of the intestinal bacteria, generating phenolic acid fission products with no antioxidant activity (Griffiths 1982). However, recent studies suggested that absorption is approximately 3-5% for the catechins, 7-24% for the flavanones, 17-52% for the flavanols, 4% for the anthocyanins and unknown for the flavones (Dwyer and Peterson 2002). Absorption of flavonoids and the rate of permeability depends on their chemical structures. It is yet to be established whether the first step in their absorption involves cleavage of the glycosides in the GI tract.

A. Stomach

There is little metabolism of flavonoids in the stomach, as has been shown that non-enzymatic deglycosylation in the human body (e.g. in the acid conditions of the stomach) does not occur (Gee *et al.,* 1998). Decomposition of procyanidin oligomers (trimer to hexamer) has been suggested to occur under the acidic conditions of the stomach, giving rise to mixtures of epicatechin monomer and dimer, which would enhance their potential for absorption in the small intestine (Spencer *et al.,* 2000). Although it is widely believed that no absorption of flavonoid takes place in the stomach, a recent study demonstrated the appearance of malvidin-3-glucoside in both portal and systemic plasma 6 min after introduction of the anthocyanins mixture into the stomach of rats (Passamonti *et al.,* 2003). The ability of anthocyanins to permeate the gastric mucosa may explain the fast kinetics of plasma appearance of anthocyanins in rats (Tsuda *et al.,* 1999) and humans (Milbury *et al.,* 2002). Absorption of quercetin aglycone in the stomach and its subsequent secretion in the bile has also been demonstrated (Crespy *et al.,* 2002). However, the contribution of the stomach to absorption of quercetin is probably limited since quercetin is present in the diet mainly as glycosides.

B. Small intestine

Deglycosylation of flavonoids is the most likely first step of metabolism during their uptake from the intestinal lumen. Several recent studies have demonstrated the hydrolysis of flavonoid glycosides prior to their absorption in the small intestine, supporting the presence of β-glucuronidase activity (Manach *et al.,* 1997, Day *et al.,*

1998, Shimoi *et al.,* 1998, Spencer *et al.,* 1999, Gee *et al.,* 2000, Walle *et al.,* 2000, Crespy *et al.,* 2001). There are two β-glycosidases present in the small intestinal cells, namely the cytosolic β-glucosidase and the lactase phlorizin hydrolyse. Both have been shown to be responsible for the hydrolysis of flavonoid glucosides (Ioku *et al.,* 1998, Sesink *et al.,* 2003, Day *et al.,* 2003). By contrast, uptake of quercetin glucoside has been demonstrated in Caco-2 cells (Walgren *et al.,* 2000), rat small intestine (Day *et al.,* 2003, Wolffram *et al.,* 2002) and in human (Paganga and Rice-Evans 1997, Hollman and Katan 1997). It therefore remains to be established if deglycosylation is a prerequisite for absorption of flavonoid glycosides. With regard to anthocyanin glycosides, there is evidence they are absorbed intact (Cao and Prior 1999, Miyazawa *et al.,* 1999).

Once the flavonoid aglycones are released, they are subjected to phase II metabolism in the small intestine upon absorption. A large number of conjugating enzymes, such as UDP-glucuronyl transferases and catechol-*O*-methyl transferases are present in the enterocytes of the small intestine (Piskula and Terao 1998, Mannisto and Kaakkola 1999). Conjugation of flavonoids by glucuronidation, methylation or a combination has been demonstrated in many studies (Shimoi *et al.,* 1998, Spencer *et al.,* 1999, Piskula and Terao 1998, Manach *et al.,* 1998, Mizuma and Awazu 1998, Crespy *et al.,* 1999, Donovan *et al.,* 1999, Kuhnle *et al.,* 2000, Kuhnle *et al.,* 2000, Donovan *et al.,* 2001). Apart from the small intestine, liver is also an important site of phase I and II metabolism, with highly active transferases capable of further modification of the flavonoid conjugates (Rice-Evans *et al.,* 2000, Piskula and Terao 1998, Donovan *et al.,* 2001).

C. Colon

Studies have suggested that only around 10-20% of dietary polyphenols are absorbed by the small intestine, with the majority reaching the large intestine (Spencer *et al.,* 1999, Kuhnle *et al.,* 2000). The colon contains approximately 10^{12} microorganisms per cm^3, resulting in enormous catalytic and hydrolytic potential. The catabolism and scission of flavonoid rings in animals was first demonstrated by Booth *et al.*, (Booth 1958) and Hackett *et al.*, (Hackett 1986). Colonic microflora catalyse the breakdown of flavonoids to low molecular weight compounds, such as phenolic acids, through many reactions including hydrolysis, dehydroxylation, demethylation, ring cleavage, decarboxylation and rapid deconjugation (Rice-Evans

et al., 2000, Spencer 2003). Fission of the flavonoid structure is dependent on their hydroxylation pattern, and can occur as shown in Fig 1.3. In addition, glycosidases secreted by the colon microflora can play an important role in the absorption of flavonoids as demonstrated by the low bioavailability of rutin (quercetin-3-rutinoside) compared to quercetin-3-glucoside in human studies. Quercetin was undetectable in plasma of ileostomy patients following oral administration (Hollman *et al.,* 1995), while its presence was detected in plasma of subjects with an intact colon (Olthof *et al.,* 2000). However, large differences exist in the composition of the colonic microflora and dietary considerations between individual, accounting for large variation in the metabolism of flavonoids.

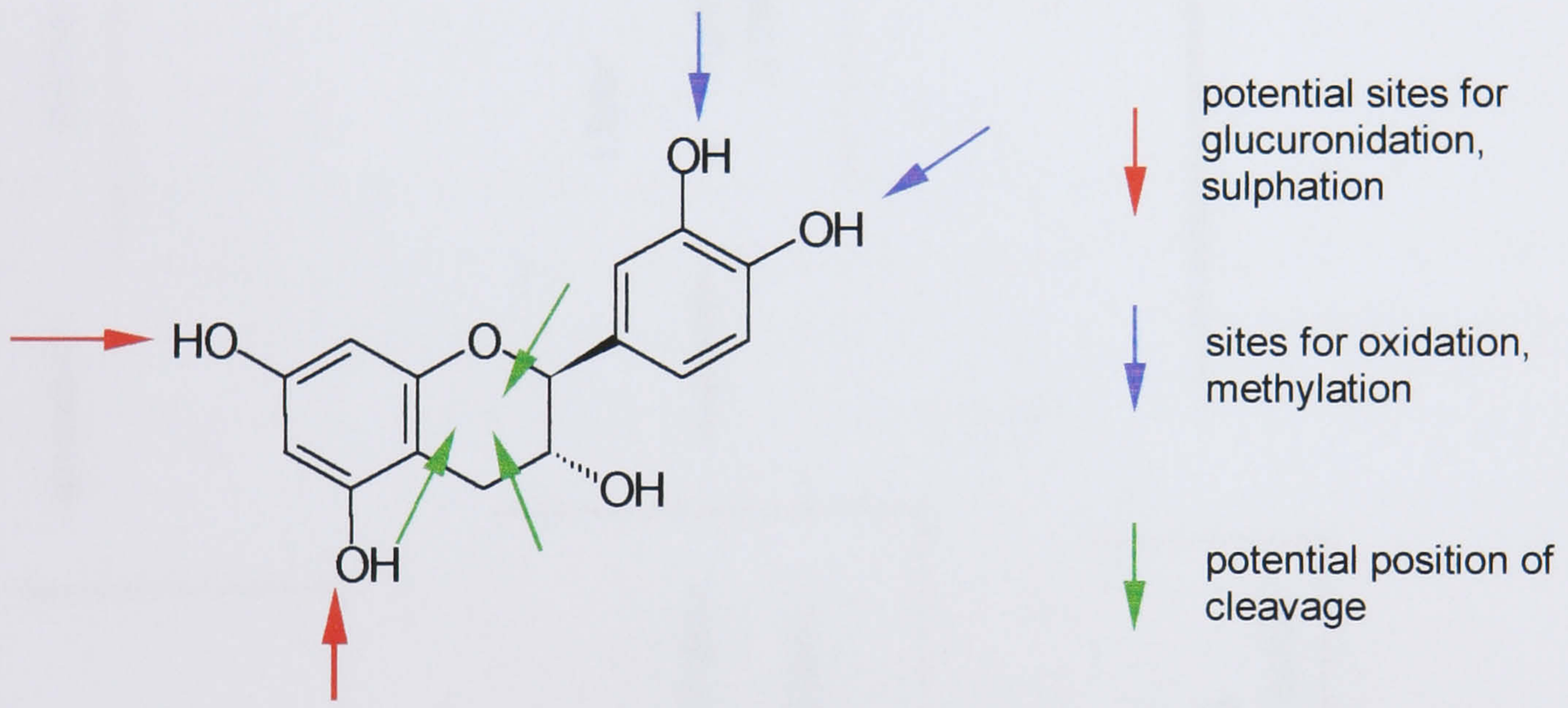


Fig 1.3 Potential sites of phase I and II metabolism of the flavanol molecule.

The various sites of biotransformation of dietary flavonoids in the body are summarised in Fig. 1.4

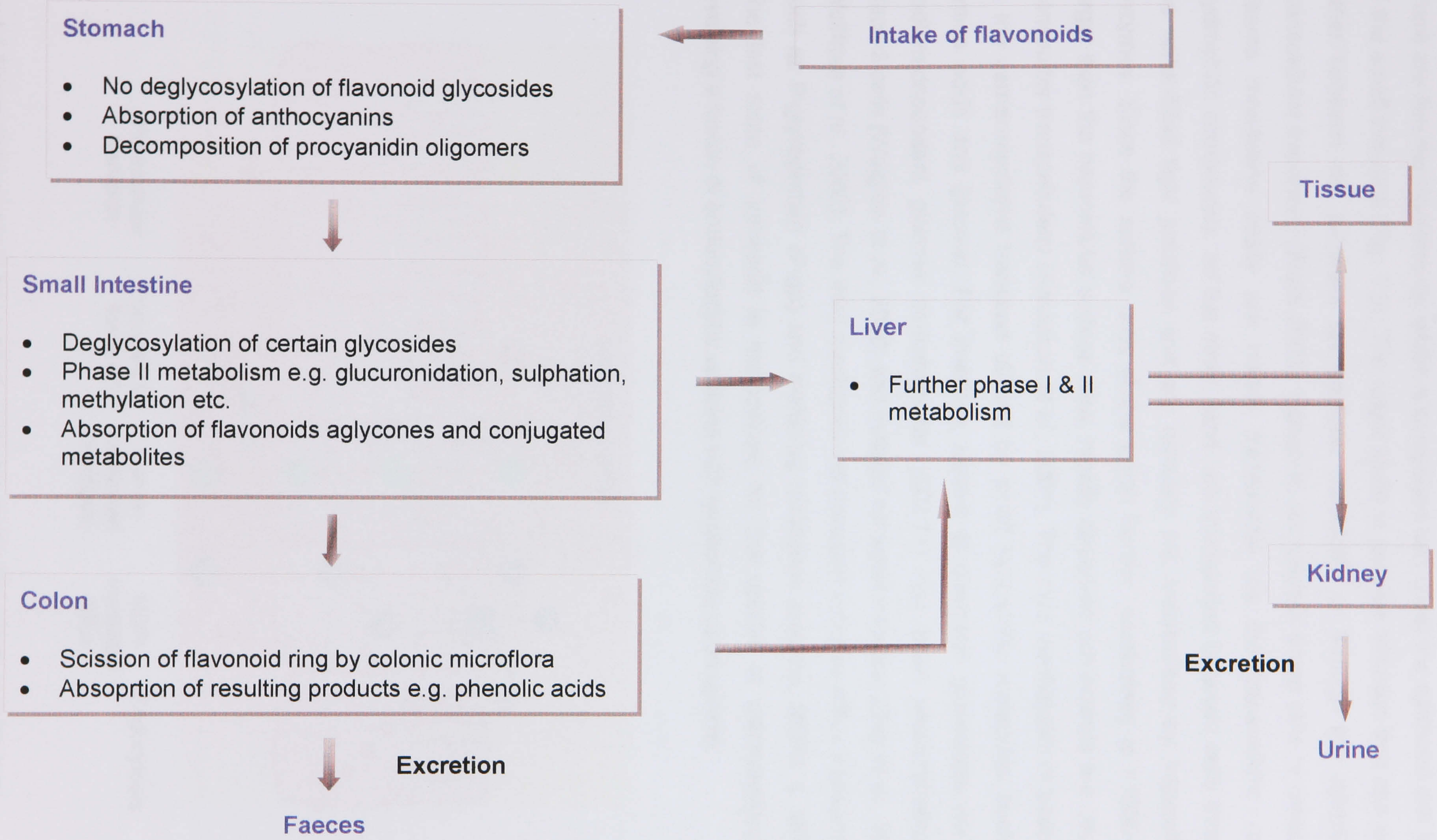


Fig 1.4 Absorption and metabolism of dietary flavonoids in the human body.

There are five mechanisms by which a compound can cross the epithelial cell layer of the small intestine (Fig. 1.5). The major route is passive diffusion that can occur either between cell junctions (paracellular transport), or through the cytoplasm (transcellular transport) (Walle 2003). Lipophilic compounds being able to cross the plasma membrane easily are mainly transported via the transcellular route. Hydrophilic compounds, on the other hand, are transported between cells through the water-filled tight junctions and are normally not metabolised by intracellular enzymes. Since the surface area of the brush border membranes is >1000-fold larger than the paracellular surface area, rapidly absorbed compounds are usually transporter transcellularly (Artursson *et al.,* 2001). The third mechanism of passage is the carrier-mediated transport utilised by small hydrophilic molecules such as amino acids and glucose. For instance, uptake of quercetin glucosides via the sodium-dependent glucose co-transporter (SGLT1) has been demonstrated in Caco-2 cells (Walgren *et al.,* 2000) and isolated rat small intestine (Day *et al.,* 2003, Wolffram *et al.,* 2002). The third mechanism of transport includes efflux transporters such as P-glycoprotein (P-gp) and multidrug resistance proteins, MRP1 & MRP2. The last route of transport is endocytosis for the uptake of macromolecules, involving a fusion of endocytocytic vesicles with lysosomes upon uptake.

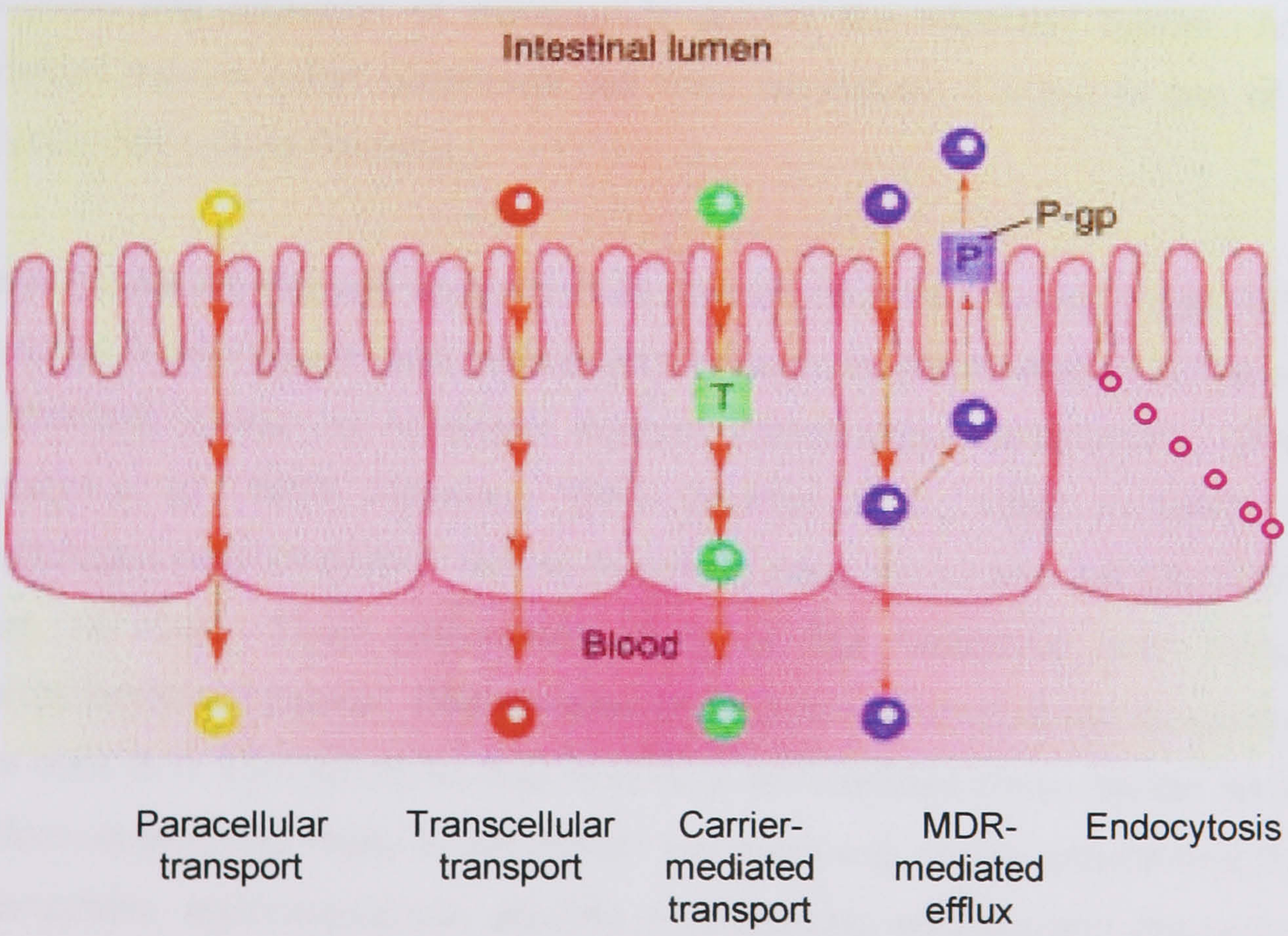


Fig 1.5 The multiple modes in transport of molecules across the intestinal epithelium.

1.1.4 Methods in absorption screening

Owing to the importance of absorption screening in the pharmaceutical industry, various models ranging from the simple computational models to complicated human *in vivo* models have been developed for evaluating drug absorption potential. The simplest models, such as structure based computational models and physiocochemical methods using artificial membranes (PAMPA), allow rapid screening of a large number of compounds. However, the bioavailability of flavonoids is largely affected by their metabolism, and as a result data obtained with cellular, animal or human model are often required for optimal reliability.

1.1.4.1 *In vitro* model

In order to successfully mimic a biological barrier like the intestinal wall with an *in vitro* cell culture system, the selection and the properties of the cell line are important. Primary cultures of enterocytes have a very poor viability and lose their polarity i.e. do not display an apical and basolateral surface upon isolation (Barthe *et al.,* 1999). Attempts have been made to develop an *in vitro* system derived from cultured intestinal epithelial cells. However, it has been proved to be difficult owing to dedifferentiation of cells when cultured as monolayers (Hidalgo 1996). As such, various (immortalised) cell lines have been developed and used to investigate diffusion and transport of compounds across the intestinal barrier. Among the different human colon carcinoma cell lines developed, Caco-2 is one of the most popular cell culture models.

Caco-2 cells are derived from a human colonic adenocarcinoma (Fogh 1977), which undergo morphological and biochemical enterocytic differentiation at the end of the proliferative phase to resemble normal, differentiated enterocytes (Pinto 1983, Hidalgo *et al.,* 1989, Artursson 1990, Meunier *et al.,* 1995, Li 2001). Cells are proliferative until 10 days in culture (reaching confluence) and are fully differentiated after 14 days. They possessed many of the functional and morphological characteristics of normal, differentiated enterocytes when cultured as confluent cells. The cells form tight junctions and develop a well-defined brush border on the apical surface expressing many brush border enzymes e.g. some cytochrome P_{450} (CYP) isoenzymes, aminopeptidase, alkaline phophastase, sucrase and phase II enzymes such as glutathione S-transferase and sulphotransferase (Walle 2003, Pinto 1983, Bohets *et al.,* 2001). Many active transport systems found in the small intestinal enterocytes have also been characterised. These include transport systems for

sugars, amino acids, bile acids, dipeptides and vitamins (Bohets *et al.,* 2001, Blais 1987, Smith 1991, Dantzig 1990).

When used as an intestinal model, cells are cultured on a porous permeable filter for at least 20 days to form a differentiated monolayer. The filter (consisting of an inert material such as polycarbonate) should have pores with a diameter of 0.4 μm in order to avoid cell migration from the apical to basolateral side. The 20-day culture time is required to obtain tight junctions, cell polarity and expression of drug efflux mechanisms such as P-gp. The integrity of monolayers can be determined by measuring the Transepithelial electrical resistance (TEER), which increases with culture time, and reaches a maximum after about 10 days in culture (Walle 2003). Resistances ranging from 150 ohm.cm^2 to up to 600 ohm.cm^2 have been reported. Experiments should be carried out with stirring to the buffer (e.g. on a rocker) to reduce the thickness of unstirred water layer (UWL), thereby minimising its effect on the apparent permeability (Avdeef 2001, Naruhashi *et al.,* 2003, Youdim 2003).

Caco-2 monolayers are a good model for compounds that are transported via the transcellular pathway (i.e. compounds with moderate lipophilicity). A good correlation between the extent of oral drug absorption in humans and rates of transport across the Caco-2 cell monolayers has been demonstrated (Lennernäs 1997, Lennernäs 1998, Yee 1997, Artursson 1991). The major advantage of the Caco-2 model is that the cells are derived from human and therefore do not suffer from interspecies differences in morphology and physiology. In addition, only small quantities of compounds (mg) are required for rapid drug absorption screening, making it a suitable model for high throughput screening. However, the tight junctions in differentiated Caco-2 monolayers are more characteristic of those in the colon than in the small intestine (i.e. higher TEER than is normally found across the small intestinal epithelium), thus low permeability are often observed (Walle 2003). The Caco-2 monolayers are devoid of the mucus layer found on the intestinal epithelium due to lack of the mucin producing goblet cells. In addition, Caco-2 cell lack some cytochrome P_{450} enzymes isoforms abundant in human intestinal tissue e.g. 3A4 (Prueksaritanont *et al.,* 1996, Cummins *et al.,* 2001) and efflux transporters (apart from P-gp), which are often under-expressed (Bohets *et al.,* 2001).

1.1.4.2 *Ex vivo* model

Models using isolated rat intestinal tissue, such as the Ussing chamber (where small sections of intestinal mucosa are clamped between two chambers containing buffer), the everted gut sac technique or the perfusion of rat intestine in paraffin oil are very popular. The permeability of compounds can be investigated in conjunction with intestinal metabolism using these models (Smith *et al.,* 1988). On top of this, regional differences in intestinal absorption can be studied easily (Naruhashi *et al.,* 2001). The major advantage of tissue-based models over cell-based model is the presence of the apical mucus layer in the former, providing a closer resemblance of *in vivo* situation (Barthe *et al.,* 1999, Zheng *et al.,* 1994). This section will be focused on the isolated rat small intestine model where the tissue is suspended in paraffin oil.

The isolated rat small intestine model was first designed and conducted by Fisher and Gardner in 1974 (Fisher and Gardner 1974). Sections of intestinal tissue were removed from rats and suspended in a chamber containing liquid paraffin at 37°C. The compound of interest is perfused through the lumen with a segmental flow of bicarbonate buffer. Absorbed fluid dropped through the paraffin to the base of the chamber and was collected at timed intervals for analysis.

The UWL is a stagnant layer consists of water, mucus and glycocalyx lining the apical side of the intestinal mucosa. It is created by incomplete mixing of the luminal contents near the intestinal mucosal surface (Levitt *et al.,* 1988), and was determined to be approximately 30-100 μm in human (Lennernäs 1998). The effect of UWL on intestinal permeability was suggested to be greater to lipophilic compounds posing as a major barrier to their uptake. When a segmented flow with O_2 is used in the rat perfusion study, the thickness of the UWL is reduced because of efficient mixing of the perfusate. This would allow prediction of flavonoid permeability with enhanced accuracy (Fisher and Gardner 1974).

1.1.4.2 *In vivo* model

Another commonly used method for predicting the extent of oral absorption of compounds is the used of *in vivo* evaluation in animals and humans. Following administration the presence of test compound and the related metabolites in plasma can be determined. *In vivo* studies provide a complete picture of absorption potential of test compounds, taking into account intestinal metabolism and intestinal physiology such as the mucus layer as well as metabolism in other parts of the GI tract and the liver. However, it is resource and labour intensive, involving analysis of a large amount of samples (Bohets *et al.,* 2001).

1.2 PROCYANIDINS

Proanthocyanidins are complex flavonoid polymers abundant in cereals, legume seeds and fruits. There are two main types of proanthocyanidins, which can be distinguished according to the substitution pattern of their B ring: procyanidins with two adjacent hydroxyl groups in the B ring and prodelphinidins with three (Santos-Buelga and Scalbert 2000).

Procyanidins are members of the flavanol family present in substantial amounts in green tea, red wine, chocolates and many fruits (Santos-Buelga and Scalbert 2000, Hammerstone *et al.,* 2000). They are oligomers and polymers of flavan-3-ols with epicatechin and catechin being the most common monomeric units. Procyanidins are synthesised by plants chemically and/or enzymatically, with an average degree of polymerisation between 3-11. Monomeric units are linked by C-C and occasionally C-O-C bonds. The most common interflavanol linkages are the C-C bonds between the C4 of one flavanol unit and the C8 or C6 of another flavanol unit (Fig. 1.6).

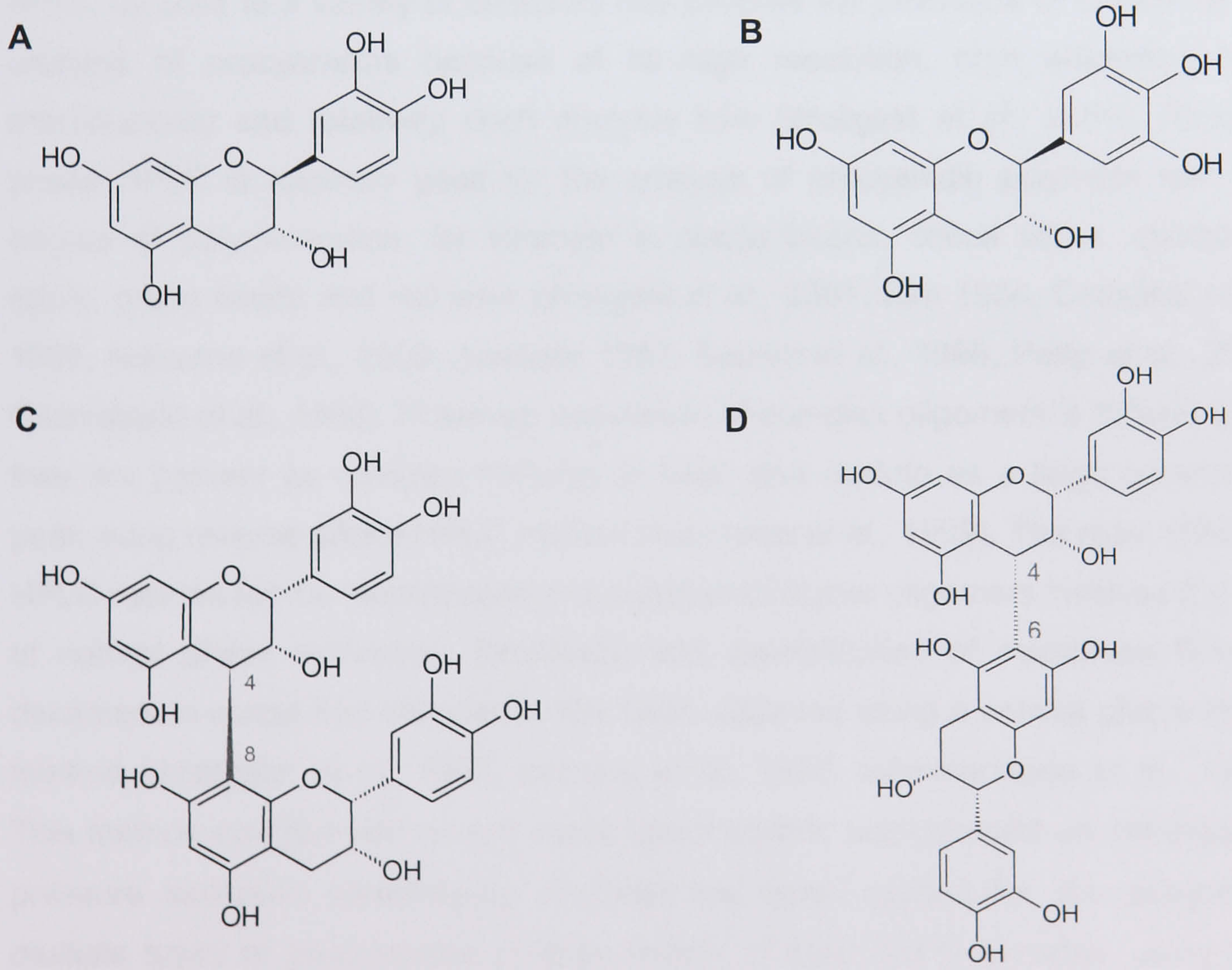


Fig 1.6 Structures of Proanthocyanidins: (A) Procyanidin, (B) Prodelphinidin, (C) B2 dimer, epicatechin-(4β-8)-epicatechin and (D) B5 dimers, epicatechin-(4β-6)-epicatechin.

Procyanidins function as antioxidants by virtue of their hydrogen-donating properties (Rice-Evans *et al.,* 1996), transition metal-chelating properties (Paganga 1996) and protein binding ability (Moini *et al.,* 2000, Riedl and Hagerman 2001). The catechol group in the B-ring is capable of donating a hydrogen atom to free radicals readily, leading to the formation of semiquinone and *o*-quinone. Furthermore, catechol groups are able to chelate metals, thereby preventing metal ion-catalysed free radical formation.

1.2.1 Dietary sources of procyanidins and their analysis

Detailed quantitative information on procyanidin content in food and their average daily intake is scarce due to the complexity of their structure and the lack of a suitable method for their estimation. This information would be useful for the understanding of the health benefits of different foodstuffs. Various methods have been applied to determine procyanidins in plants and foods, including the use of folin reagent (Swain 1959), the formation of a coloured adduct with aromatic aldehydes in strongly acidic media (Deshpande *et al.,* 1986, Treutter 1989), and the determination of anthocyanidin following depolymerisation (Porter 1986). Recently, HPLC coupled to a variety of detectors has become the technique of choice for the analysis of procyanidins because of its high resolution, high efficiency, high reproducibility and relatively short analysis time (Wollgast *et al.,* 2001). Reverse phase HPLC is routinely used for the analysis of procyanidin polymers with low degree of polymerisation, for example in cocoa beans, cocoa liquor, chocolate, apple, grape seeds and red wine (Wollgast *et al.,* 2001, Kim 1984, Osakabe *et al.,* 1998, Natsume *et al.,* 2000, Jaworski 1987, Suarez *et al.,* 1996, Peng *et al.,* 2001, Oszmianski *et al.,* 1988). However, separation of complex oligomers is difficult since they are present as complex mixtures in food, and coelute as a large unresolved peak using reverse phase HPLC method (Adamson *et al.,* 1999). The most effective HPLC method for the identification and isolation of higher oligomers involves the use of normal phase technique. Separation and quantification of monomers through decamers in cocoa and chocolates has been achieved using a normal phase HPLC method (Adamson *et al.,* 1999, Lazarus *et al.,* 1999, Hammerstone *et al.,* 1999). This method coupled with on-line mass spectrometric analysis with an atmospheric pressure ionisation electrospray chamber has been applied for the analysis of multiple types of procyanidins in large variety of food and beverages, using both ultraviolet and fluorescence detectors for increased selectivity and sensitivity (Lazarus *et al.,* 1999, Hammerstone *et al.,* 1999). This has enabled the extraction

and purification of oligomers that are used as standards in routine quantification of procyanidins.

Amongst the limited information on the distribution and content of procyanidins in food, large discrepancies are observed according to authors, which can be explained by the differences in the analytical method used. Other factors influencing the procyanidin content in food include stage of ripeness, variety, part of the fruit consider and the processing method into foodstuffs. Table 1.3 illustrates the procyanidin content in different foodstuffs and beverages commonly consumed in Western diets.

Sources	*Procyanidin content*	*Analytical procedure*	*References*
	Fruits and berries (mg per 100g)		
Apple	9.8 – 42.9	HPLC - DMACA	1
Blackberry	9 – 11	HPLC	4
Blueberry	5.3	HPLC - DMACA	1
Cherry	12.0	HPLC - DMACA	1
Grape, red	3.4	HPLC - DMACA	1
Grape, white	1.8	HPLC - DMACA	1
Peach	8.6	HPLC - DMACA	1
Pear	0.7 - 12	HPLC	4
Plum	49.7	HPLC - DMACA	1
Raspberry	6.2	HPLC - DMACA	1
Redcurrant	5.1	HPLC - DMACA	1
Strawberry	4.91	HPLC	
Sweet cherry	10-23	HPLC	
	Juices and drinks (mg/L)		
Apple juice	ND – 298	HPLC	4
Beer	6.4	HPLC - DMACA	1
Cranberry juice	114-151	NP-HPLC	2
Grape juice	3.5 – 46	HPLC	4
Tea, black	268.0	HPLC - DMACA	1
Tea, green	438.3	HPLC - DMACA	1
Wine, red	196 - 235	NP-HPLC	2
Wine, white	20.0	HPLC - DMACA	1
	Others (mg per 100g)		
Broad bean	154.5	HPLC - DMACA	1
Cocoa bean	260-1200	Not given	3
Dark chocolate	379-490	NP-HPLC	2
Pinto bean	7.4	HPLC - DMACA	1

Table 1.3 Procyanidin content in different foodstuffs (expressed as mg/100g fresh weight) and beverages (mg/L) commonly consumed in Western diets.
Abbreviations: HPLC-DMACA, HPLC and post-column derivatisation with DMACA, NP-HPLC, normal phase HPLC, RP-HPLC, reverse phase HPLC. [1] de Pascual-Teresa *et al.,* 2000; [2] Hammerstone *et al.,* 2000; [3] Porter *et al.,* 1991; [4]Santos-Buelga *et al.,* 2000.

In a recent study the daily catechin intake was reported to be 50 mg/d in the Dutch population, with tea being the primary source for all ages, and chocolate an important source in children (Arts *et al.,* 2001, Arts *et al.,* 2001). No information has yet been obtained for the daily intake of epicatechin.

1.2.1.1 Procyanidins in chocolate (*Theobroma cacao*)

Humans have been consuming chocolates for thousands of years but their beneficial properties were not recognised until the discovery of polyphenolic antioxidants. Cocoa beans are extremely rich in polyphenols in the forms of procyanidin oligomers, comprising 12-18% of dry weight of the whole bean (Porter 1991). The polyphenols in cocoa beans are stored in the pigment cells of the cotyledons (polyphenol-storage cells). There are three groups of polyphenols identified in cocoa beans, namely the catechin or flavan-3-ols (37%), anthocyanins (4%) and proanthocyanidins (58%). The most abundant catechin is (–)-epicatechin (up to 35% of polyphenol content) with small amounts of (+)-catechin as well as traces of (+)-gallocatechin and (–)-epigallocatechin present (Kim 1984). Procyanidins are mostly flavan-3-ols that are 4→8 or 4→6 bound to condensed dimers, trimers or oligomers with epicatechin as the main extension subunit (Wollgast and Anklam 2000). The contribution of chocolate to the total intake of catechins in a representative sample of the Dutch population has recently been estimated. While tea was the most important source of catechins (consisted 55% of total intake) chocolate contributed 20% to the total intake. It was further suggested that in younger age groups, in which chocolate is consumed more regularly than tea, chocolate may be an even more important source of catechins (Arts *et al.,* 1999).

1.2.2 Absorption and metabolism

A. Oral Cavity

The first proteins encountered by procyanidins once ingested are those in the palate. The macromolecules in saliva consist largely (~70%) of the proline-rich proteins (PRPs). These salivary PRPs bind procyanidins very strongly due to their highly randomised, open-chain conformation. Complexation occurs via the hydrophobic sites on the protein surface (Hagerman 1993, Haslam 1996). Many of the complexes with high molecular weight procyanidins are not readily dissociated

and are stable throughout the digestion process. However, it was observed by Spencer *et al.*, (Spencer *et al.,* 2001) that incubation of procyanidins (dimer – hexamer) in human saliva for up to 30 minutes did not result in any modification of the compounds, with >99% recovery of procyanidins. It is however important to take this into consideration when investigating the subsequent biological actions of procyanidins.

B. Small intestine

A recent study has demonstrated the absorption and metabolism of catechin and epicatechin in the small intestine using an isolated rat small intestine perfusion model (Kuhnle *et al.,* 2000). After perfusion of the jejunum with catechin and epicatechin, glucuronidated metabolites as well as both *O*-methylated and *O*-methylated-glucuronides were detected on the serosal side. The structures of these metabolites are depicted in Fig. 1.7. By contrast, the major metabolite after perfusion of the ileum with catechin and epicatechin was the unmodified flavan-3-ol itself, which was transferred much more readily than the modified flavan-3-ol (Kuhnle *et al.,* 2000). It was proposed that the extent of glucuronidation was dependent on the number of hydroxyl group present on the flavonoids. Catechin and epicatechin with the catechol structure were absorbed predominantly as glucuronides. Another factor governing the extent to which procyanidins are absorbed by the intestine is the degree of polymerisation. Procyanidins with high degree of polymerisation are unlikely to be absorbed by the small intestine in their native forms due to their large sizes. This is supported by the evidence that only procyanidin dimers and trimers, but not polymers were absorbed through an intestinal epithelium cell monolayer *in vitro* (Deprez *et al.,* 2001). When the absorption of procyanidin dimer was studied using an *ex vivo* isolated rat small intestine model, <1% of procyanidin dimers B2 and B5 were absorbed across the jejunum or ileum of the rat small intestine. Low levels of O-methylated dimer (~3.2%) were also detected, but the majority of the perfused dimer (95.8%) was recovered as unmetabolised / unconjugated epicatechin on the serosal side (Spencer *et al.,* 2001). Together these observations suggest procyanidin dimers, and possibly trimers can be absorbed across the small intestine either as the intact compounds or as the monomeric units.

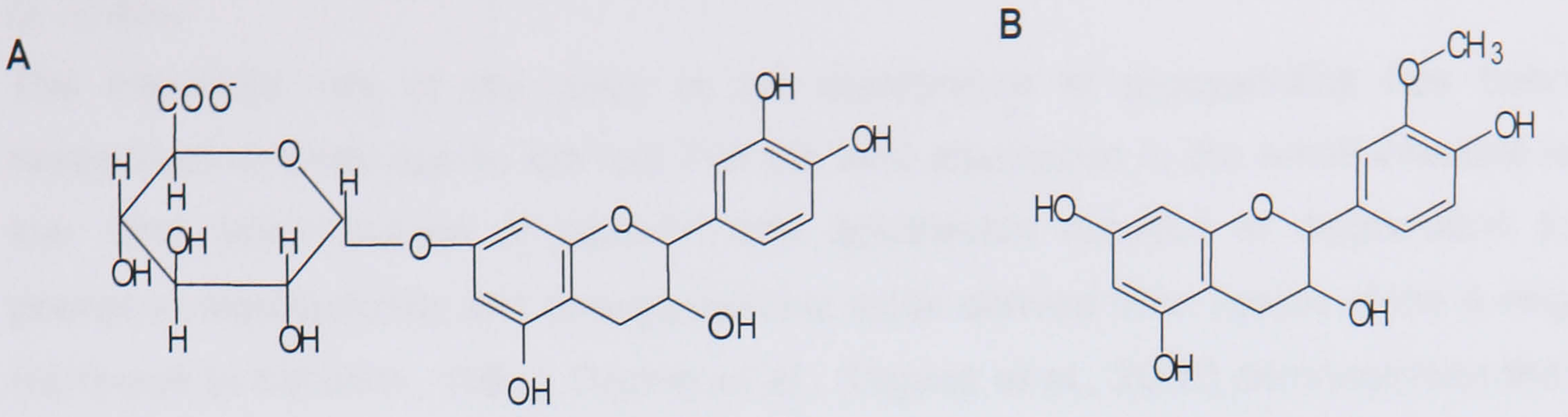


Fig 1.7 Structure of conjugates and metabolites of epicatechin. A) epicatechin-7-glucuronide; B) 3′-O-methyl epicatechin; C) 4′-O-methyl epicatechin; D) 3′-O-methyl-(7-glucuronide)-epicatechin.

C. Gastric lumen

While the absorption of procyanidin oligomers in the small intestine requires further research, it has been suggested that pre-absorption event in the stomach may influence their subsequent absorption in the small intestine. Procyanidin oligomers isolated from chocolate may be hydrolysed into mixtures of epicatechin monomers and dimers in the gastric lumen under acidic conditions (Spencer *et al.,* 2000). The decomposition was shown to be rapid with almost complete loss of oligomers after about 3 hours i.e. the residence time of food in stomach. However, contradictory results were obtained from a later human study examining gastric depolymerisation of procyanidin *in vivo*. When subjects consumed procyanidins (monomers up to pentamers) in the form of cocoa beverages, procyanidins were found to be stable in the stomach environment during gastric transit. The difference is probably due to the fact that *in vivo* gastric pH is buffered by food consumption and was increased to 5.4 after ingestion of the cocoa beverage, whereas the pH used in the *in vitro* study was around 2, i.e. under basal conditions (Rios *et al.,* 2002). As such, consideration needs to be given to the food matrix, which may influence the pH environment of the procyanidins, and ultimately their decomposition (Spencer 2003).

D. Colon

The important role of the colon in the metabolism of procyanidins has been recognised recently due to the fact that the total absorption in the small intestine is low. Oral administration of catechin and epicatechin resulted in degradation to phenyl γ-valerolactones and phenylpropionic acids derived from fission of the A-ring (reviewed in Scheline, 1991). Déprez *et al.*, (Deprez *et al.,* 2000) demonstrated that procyanidin polymers were degraded by a human colonic microflora grown *in vitro* anaerobically into low-molecular weight aromatic acids, namely 3,4-dihydrophenylacetic acid, 3-hydroxyphenylacetic acid, homovanillic acid and their conjugates. Absorption of these compounds was confirmed by the observation that such metabolites have been detected in human plasma and urine after a single ingestion of green tea (Li *et al.,* 2000). Another study has demonstrated that feeding rats and mice with a radiolabelled mixture of procyanidins (containing oligomeric procyanidins, (+)-catechin and (-)-epicatechin) resulted in the identification in urine and faeces of some ill-defined procyanidins and low-molecular weight metabolites. These include various phenolic acids, ethylcatechol and hippuric acid – the glycine conjugate of benzoic acid produced through the action of intestinal bacteria (Harmand 1978).

Flavonoids can actually serve a beneficial role in the GI tract regardless of their absorption. Reactive oxygen species (ROS) are produced in the GI tract via oxidation of dietary lipids and transitional metal-catalysed Fenton reaction (Riedl and Hagerman 2001, Halliwell *et al.,* 2001). Ingested food frequently contains iron ions, usually as insoluble Fe(III) salts. Ferric ion is solubilised by gastric acid, which can then be reduced by ascorbate and other reductants to Fe^{2+}. Thus the GI tract may be target for damage by hydroxyl radical ($OH^{\bullet}$) generated by Fenton chemistry from ascorbate/Fe^{2+} mixtures (Kadiiska *et al.,* 1995). The substrate of the reaction, hydrogen peroxide, has been detected in several foods, and high levels (>100 μM) are found in certain beverages such as teas, ground coffee and especially instant coffees (Riedl and Hagerman 2001, Halliwell *et al.,* 2001). Polyphenols can offer protection against the detrimental effects of ROS to the contents or lining of the gut lumen. In addition, by scavenging ROS, polyphenols can spare low molecular weight, bioavailable antioxidants (Riedl and Hagerman 2001).

Once procyanidins or their breakdown products have crossed the gut barrier, they reach the liver and are further metabolised.

Source and dose of procyanidin	Analytical method	Plasma concentration of metabolites detected (μM)					T (max)	Reference
		Free	Glucuronides	Sulphates	Sulpho-glucuronides	Dimer B2		
80 g dark chocolate	Enzyme treatments + HPLC	EC 0.7	-	-	-	-	2 – 3 h	1
27 g semisweet [1] chocolate	Enzyme treatments + HPLC	EC 0.133	-	-	-	-	2 h	2
35 g semisweet [1] chocolate	Enzyme treatments + HPLC	EC 0.258	-	-	-	-	2 h	2
80 g semisweet [1] chocolate	Enzyme treatments + HPLC	EC 0.355	-	-	-	-	2 h	2
80 g semisweet chocolate	HPLC-ECD	EC 0.257	-	-	-	-	2 h	3
35 g chocolate[2]	Enzyme treatments + LC-MS	EC 0.15	EC 0.78	EC 1.11 Me-EC 0.95	EC 1.07 μM Me-EC 0.71	-	2 h	4
35 g cocoa[2]	Enzyme treatments + LC-MS	EC 0.22	EC 0.91	EC 1.14 Me-EC 1.00	EC 1.19 μM Me-EC 0.46	-	2 h	4
26.4 g cocoa[3]	LC-MS/MS	EC 5.92 CA 0.16	-	-	-	0.041	2 h	5

Table 1.4A The bioavailability of procyanidins following human feeding studies of chocolate and cocoa.

Source and dose of procyanidin	Analytical method	Plasma concentration of metabolites detected (μM)					T (max)	Reference
		Free	Glucuronides	Sulphates	Sulpho-glucuronides	Dimer B2		
50 mg EC / kg body weight	Enzyme treatments + LC-MS	EC 1.2	EC 10.7 Me-EC 4.5	EC 1.7 Me-EC 4.0	EC 7.0 Me-EC 11.5	N.A.	2 h	6
50 mg EC / kg body weight [4]	Enzyme treatments + HPLC	EC 3.0	EC 25.2 Me-EC 7.4	EC 1.0 Me-EC 1.3	Me-EC 4.4	N.A.	1h	7
250 mg EC / kg body weight [4]	Enzyme treatments + HPLC	EC 13.3 Me-EC 0.9	EC 52.1 Me-EC 5.9	Me-EC 7.6	Me-EC 23.2	N.A.	1 h	7
100 mg CA / kg body weight	HPLC + NMR	CA 0.77	CA 6.90 Me-CA 2.86	-	-	N.A.	2 h	8
100 mg EC / kg body weight	HPLC + NMR	EC 1.09	EC 15.56 Me-EC 3.76	-	-	N.A.	2 h	8
0.25 % catechin diet [5]	Enzyme treatments + HPLC	CA 40.3 (mainly glucuronidated derivatives)				N.A.	4 h	9
0.25 % catechin diet [6]	Enzyme treatments + HPLC	CA 27.6 (mainly glucuronidated derivatives)				N.A.	4 h	9
1 g cocoa powder /kg body weight [7]	Enzyme treatments + LC-MS	EC 0.3	EC 6.9 Me-EC 2.0	N.D.	EC 1.5 Me-EC 1.4	-	0.5 – 1 h	10
50 mg CA / kg body weight [8]	Enzyme treatments + LC-MS	CA 0.19 Me-CA 0.03	CA 43.6 Me-CA 11.6	CA 1.84 Me-CA 8.5	CA 21.1 Me-CA 0	N.A.	1 h	11

50 mg EC / kg body weight [8]	Enzyme treatments + LC-MS	EC 6.48 Me-EC 0.87	EC 39.9 Me-EC 14.9	EC 0 Me-EC 41.2	EC 41.9 Me-EC 22.1	N.A.	1 h	11
50 mg CA + 50 mg EC / kg body weight [8]	Enzyme treatments + LC-MS	CA 0.15 Me-CA 0 EC 7.73 Me-EC 0.82	CA 44.2 Me-CA 11.7 EC 36.0 Me-EC 11.9	CA 9.89 Me-CA 13.6 EC 0 Me-EC 54.1	CA 0 Me-CA 0 EC 33.1 Me-EC 11.8	N.A.	1 h	11
50 mg dimer B2 / kg body weight	Sulphatase treatment + LC/MS	N.D.	-	EC 0.22 Me-EC 0.66	-	Sulphated 0.5	0.5 h	12

Table 1.4B The bioavailability of procyanidins following rat feeding studies of procyanidin-rich food.
CA – catechin; EC – epicatchin; Me-CA – methylated catechin; Me-EC – methylated epicatechin. [1] each 27 g chocolate portion contained 46 mg epicatechin and a total of 186 mg procyanidins. [2] values were obtained 2 h after intake of chocolates and cocoa, both containing the same amount of procyanidins. [3] 26.4 g cocoa providing 323 mg monomers and 256 mg dimers. Values were obtained 2h after intake of cocoa. [4] Values obtained 1h after EC administration via intragastric intubation. [5] Diet contained 62.5 mg catechin per day for 1 day. [6] Diet contained 62.5 mg catechin per day for 14 days. [7] 1 g cocoa powder contained 780 mg epicatechin, 305 mg catechin and 252 mg procyanidin dimer B2. Values obtained 1 h after cocoa powder intake. [8] Values are amount of metabolite detected per hour up to 5 h post-administration.

References: [1] (Richelle *et al.*, 1999); [2] (Wang *et al.*, 2000); [3] (Rein *et al.*, 2000); [4] (Baba *et al.*, 2000); [5] (Holt *et al.*, 2002); [6] (Piskula and Terao 1998); [7] (Da Silva *et al.*, 1998); [8] (Harada *et al.*, 1999); [9] (Manach *et al.*, 1999); [10] (Baba *et al.*, 2000); [11] (Baba *et al.*, 2001); [12] (Baba *et al.*, 2002).

1.2.2.1 *In vivo* studies

Recently, many feeding studies have been carried out to investigate the bioavailability of flavan-3-ols and procyanidins derived from cocoa beans. A summary of the results from various studies is given in Tables 1.4A and B. Results from many of the studies suggest that catechin and epicatechin are extensively metabolised, with the glucuronide-, sulphate-, sulphoglucuronide-conjugates of non-methylated or methylated catechin or epicatechin being the major metabolites present in plasma of rat and human. There is also evidence that the bioavailability of epicatechin is higher than that of catechin in rats (Baba *et al.,* 2001). In a recent study the structures of the major metabolites of epicatechin were identified from plasma and urine of human and rat using LC-MS and NMR. The authors suggested that glucuronidation of epicatechin occurs at the 3' position of the B ring in human, whereas in rat glucuronidation occurs at the 7 position of the A ring (Natsume *et al.,* 2003). The difference observed in the study was probably due to different distribution of the various isoforms of UGT enzymes in humans and rats.

A sequence of metabolic pathway of orally administered epicatechin in rats was proposed by Piskula and Terao (Piskula and Terao 1998). After feeding rats with epicatechin (172 μmol/kg body weight), the epicatechin metabolites in plasma as a function of time were determined. Majority (90%) of plasma epicatechin metabolites contained the glucuronide moiety shortly after its administration, indicating that glucuronidation takes place almost immediately after reaching the small intestine. The level of epicatechin glucuronide decreased at the highest rate amongst all metabolites, probably due to further metabolism and excretion via bile or urine. The final products formed from the absorbed epicatechin are likely to be the methylated metabolites, since they remained in the blood circulation for the greatest amount of time. Therefore, it was proposed that the first metabolic event happening in the alimentary tract is glucuronidation of epicatechin, facilitating its absorption. Glucuronidated epicatechin then enters the liver and kidney via the portal vein, where sulphation (liver) and methylation (liver and kidney) occurs (Piskula and Terao 1998).

The transfer and metabolism of procyanidin oligomers remains unclear. Earlier *in vivo* studies focused on the monomeric units and showed that following ingestion of cocoa-derived product, epicatechin and its metabolites were detected in rat and human plasma (Baba *et al.,* 2000, Baba *et al.,* 2000, Wang *et al.,* 2000). No information was obtained for higher procyanidin oligomers from these studies.

Currently studies are focusing on the absorption of procyanidin dimers after administration of the pure compounds or procyanidin-rich food. The presence of procyanidin B2 in plasma of rats following oral administration of the purified compound was demonstrated in two recent studies (Baba *et al.,* 2002, Tanaka *et al.,* 2003). This was supported by the detection of procyanidin B2 in human plasma after the consumption of cocoa (Holt *et al.,* 2002). Evidence for absorption of procyanidin B3 is more confusing, with one study demonstrating its presence in rat plasma (Tanaka *et al.,* 2003) while it was suggested not to be absorbed in another study (Donovan *et al.,* 2002). It is unlikely that procyanidin oligomers with a degree of polymerisation higher than 2 would be absorbed into the circulation. Nevertheless, the presence of these higher oligomers in circulation cannot be ruled out without further study.

Many *in vitro* studies have shown that cocoa polyphenols in the form they are consumed are potent antioxidants in chemical (Pannala *et al.,* 1997, Arteel *et al.,* 2000, Oldreive *et al.,* 1998) and in biological models (Kondo *et al.,* 1996, Lotito and Fraga 1998, Osakabe *et al.,* 2002). However, since structural modification may alter their biological activity, further study is required to investigate the effect of metabolism on the properties of these polyphenols. Although conjugation and methylation of the phenolic moiety results in a decrease in its antioxidant capacity (Rice-Evans *et al.,* 1996), it has been reported that consumption of cocoa product can enhanced the antioxidant capacity in rat (Baba *et al.,* 2000) and human plasma (Wang *et al.,* 2000, Rein *et al.,* 2000), as well as increasing the resistance of human LDL to oxidation (Osakabe *et al.,* 2001). The metabolites, catechin-5-*O*-glucuronide and 3'-*O*-methyl-epicatechin, purified from rat urine retained antioxidant activity as scavengers of superoxide anion radicals (Harada *et al.,* 1999). Moreover, methylation of epicatechin did not affect its protection against H_2O_2-induced cytotoxicity in cortical neurons and dermal fibroblasts (Spencer *et al.,* 2001, Spencer *et al.,* 2001), suggesting that hydrogen-donating antioxidant activity is not the primary mechanism of protection. However, epicatechin-5- and epicatechin-7-*O*-glucuronides had no significant protection against peroxide-induced loss in neuronal or fibroblast viability. Furthermore, they were unable to offer protection against peroxide-induced caspase-3 activation in both cell models, probably due to their inability to access the cells (Spencer *et al.,* 2001). Further studies are thus required to determine the influence of metabolism on the pharmacokinetics of procyanidins, and their subsequent biological activities before conclusions can be drawn on their potential properties in tissues.

Introduction

1.2.3 Antioxidant properties of procyanidins

Studies have shown that procyanidins are potent antioxidants *in vitro* against ROS such as superoxide anion, hydroxyl radicals (Moini *et al.,* 2000), and reactive nitrogen species (RNS) such as nitric oxide and peroxynitrite (Arteel *et al.,* 2000, Virgili *et al.,* 1998). Their ability to scavenge reactive species can be attributed to their electron-donating properties, resulting in the formation of relatively stable radicals, thereby terminating radical propagation. However, procyanidins can act as antioxidants through other mechanisms, which are discussed below.

1.2.3.1 Metal chelating properties of procyanidins

The catechol groups of procyanidins can be chelators of Fe(III), Al(III) and Cu(II), and the resulting complexes are easily precipitated at neutral pH (reviewed in Santos-Buelga and Scalbert 2000). Complexation of Fe(III) and Al(III) by procyanidins from wine and tea inhibits the absorption of the metal ions across the gut barrier (Santos-Buelga and Scalbert 2000, Cook *et al.,* 1995, Hurrell *et al.,* 1999, Drewitt *et al.,* 1993), and was suggested to play a potential role in metal-overload diseases (Mira *et al.,* 2002). Ingested food frequently contains iron ions, which may lead to generation of hydroxyl radical ($OH^{\bullet}$) by Fenton chemistry, and target the stomach, duodenum and upper small intestine (Halliwell *et al.,* 2001). Procyanidins might inhibit free radical formation and the propagation of free radical reactions through the chelation of iron ions. Furthermore, the ability of catechin to prevent iron-mediated lipid peroxidation in iron-loaded hepatocytes cultures has been demonstrated (Morel *et al.,* 1993). The cytoprotective activity of catechins was ascribed to their ability to chelate iron, as shown by the relationship between their ability to remove iron from the cells and their cytoprotective activity. Direct evidence for the interaction of procyanidins with Cu^{2+} ions is lacking. However, previous study has shown that flavonoids such as quercetin and kaempferol are capable of chelating Cu^{2+} ions, and this chelation probably occurs through the catechol structure (Brown *et al.,* 1998). Since catechin and epicatechin posses the catechol structure, it is speculated that they have the ability to chelate Cu^{2+} ions and therefore to inhibit free radical-mediated reactions.

1.2.3.2 Protein binding properties of procyanidins

The characteristic astringency of procyanidins is caused by their abilities to precipitate salivary proteins in the oral cavity. Proteins with an open, random coil conformation and those that are rich in proline, such as collagen, gelatin and

salivary proteins, can form complexes with procyanidins readily (Hagerman 1993, Haslam 1996). The principal interactions are hydrophobic effects and hydrogen bonding established between the phenolic groups (as proton donors) and the carbonyl groups of the peptide bonds (as proton acceptors) (Luck *et al.,* 1994, Charlton *et al.,* 1996). The interaction of procyanidins with salivary PRPs can result in the reduction of net protein utilisation (Hussein 1985) and bioavailability of procyanidins. The procyanidin monomers, catechin and epicatechin, were shown to bind PRPs in a recent study, whereas monomers and dimers were unable to form insoluble aggregates with bovine serum albumin (BSA) (de Freitas and Mateus 2001).

One of the underlying mechanisms for the observed biological effects of procyanidins could be interaction with cellular proteins, namely, modulation of enzyme activity. Procyanidins isolated from French maritime pine bark can bind to the native structure of xanthine oxidase and inhibits its activity, which was restored upon dissociation (Moini *et al.,* 2000). Xanthine oxidase is capable of oxidising its substrate hypoxanthine in the presence of O_2, leading to the generation of xanthine, (superoxide radical) $O_2^{\bullet-}$ and H_2O_2. As a result, inhibition of xanthine oxidase would lead to the inhibition of ROS formation such as $O_2^{\bullet-}$ and H_2O_2. Furthermore, catechin and procyanidins from red wine have been shown to bind apo A-I (the major protein in human plasma HDL) in human plasma. This binding was further suggested to activate lecithin:cholesterol acyltransferase, resulting in the reverse transport of cholesterol from tissues to the liver for excretion (Brunet *et al.,* 2002).

1.2.4 Biological activities of Procyanidins

Procyanidins have attracted increased attention in the field of health and medicine due to their potent antioxidant capacity, and thus their possible role in the protection of various diseases involving oxidative stress. Recently, many studies have demonstrated other biological activities of procyanidins that may result in beneficial implications on human health.

One of the biological activities described is the ability of procyanidins to modulate immune functions, as demonstrated by Sanbongi *et al.*, (Sanbongi *et al.,* 1997) that cacao liquor polyphenols can reduce the expression of interleukin-2 mRNA in human lymphocytes via inhibition of ROS. In another study cocoa procyanidins were found to modulate the production of cytokines interleukin (IL)-1β and IL-4, both play

a role in the onset of inflammation and the latter also enhances IgG production by B cells (Mao *et al.,* 2000). Although the mechanism by which cocoa polyphenols modulate immune response remains unclear, studies have shown that they can inhibit intracellular ROS such as H_2O_2 and O_2^- (Sanbongi *et al.,* 1997). Reactive oxygen species may activate NF-KB, which subsequently mediates transcription and secretion of many cytokines. This ultimately leads to the alteration of cellular cytokine profiles and affects the responses to carcinogens and inflammatory mediators. Further studies on the immune modulation of cocoa procyanidins using *in vivo* model may proved to be advantages in the treatment of human diseases involving activation of the immune system, e.g. arthritis and eczema (Mao *et al.,* 2000).

Apart from modulation of immune funtion, the cardioprotective effects of procyandins have also been studied recently. For instance, procyanidin fractions (monomer through decamer) were demonstrated to induce endothelium-dependent relaxation (EDR) in rabbit aortic ring *in vitro* (Karim *et al.,* 2000). In addition, tetramers and higher polymers of epicatechin also have the capacity to activate endothelial nitric oxide synthase (NOS). These findings demonstrated that polymeric procyanidins could cause an EDR that is mediated by activation of NOS (Karim *et al.,* 2000). Another study looked at the effect of procyanidins on platelet activation and function. When selected cocoa procyanidin fractions were added to whole blood *in vitro*, platelet activation response to epinephrine was suppressed. Suppression of platelet activation was also demonstrated in whole blood after cocoa consumption (Rein *et al.,* 2000). Furthermore, platelet function was inhibited after supplementation of cocoa flavanol and procyanidins for 28 days in human (Murphy *et al.,* 2003). Since blood platelets play a major role in cardiovascular disease and thrombosis, procyanidins may offer cardioprotection via the suppression of platelet activities.

Recently studies have been focusing on the potential cellular activities of procyanidins, in particular their abilities to interact with cellular signaling pathways. Epicatechin has been shown to reduce the neurotoxicity induced by oxidized low-density lipoprotein (Schroeter *et al.,* 2001) via modulation of the JNK signal transduction pathway, which contributes to apoptotic response upon activation. Interactions between epicatechin and caspase-3 (a marker for apoptosis) have also been demonstrated, resulting in protection of human dermal fibroblasts and primary cortical neurons against H_2O_2-induced cytotoxicity (Spencer *et al.,* 2001, Spencer *et al.,* 2001). The *in vivo* metabolite 3'-*O*-methyl epicatechin was also shown to offer

similar protection to dermal fibroblasts (Spencer *et al.,* 2001). Furthermore, the ability of catechin to protect fibroblasts against ROS-mediated damage (Subirade *et al.,* 1995), and against H_2O_2-induced damage to cultured rat hepatocytes (Nagata *et al.,* 1999) have also been reported.

1.3 NITRATE AND NITRITE

Nitrate (NO_3^-) and nitrite (NO_2^-) are naturally occurring, water soluble anions. Nitrate is present abundantly in soil and water and is taken up by plants as a major source of nitrogen (Hill 1991). Until recently, nitrate was perceived as a purely harmful dietary component that is associated with infantile methaemoglobinaemia and increasing risk of gastric cancer (McKnight *et al.,* 1999). The daily dietary exposure of the general population to nitrate and nitrite was estimated to be 52 mg/day (Ysart *et al.,* 1999) and 0.3-0.9 mg/day, respectively (Ministry of Agriculture 1987). Green leafy vegetables such as lettuce and spinach contributed approximately 70% to the total dietary exposure of nitrate (Ysart *et al.,* 1999). Other dietary sources include root vegetables such as beetroot, and both municipal and well water (Ministry of Agriculture 1987). Nitrite is a widely used food preservative and the main dietary sources of nitrite include canned meat and sausages (Cammack *et al.,* 1999).

1.3.1 The fate of nitrate and nitrite in the human body

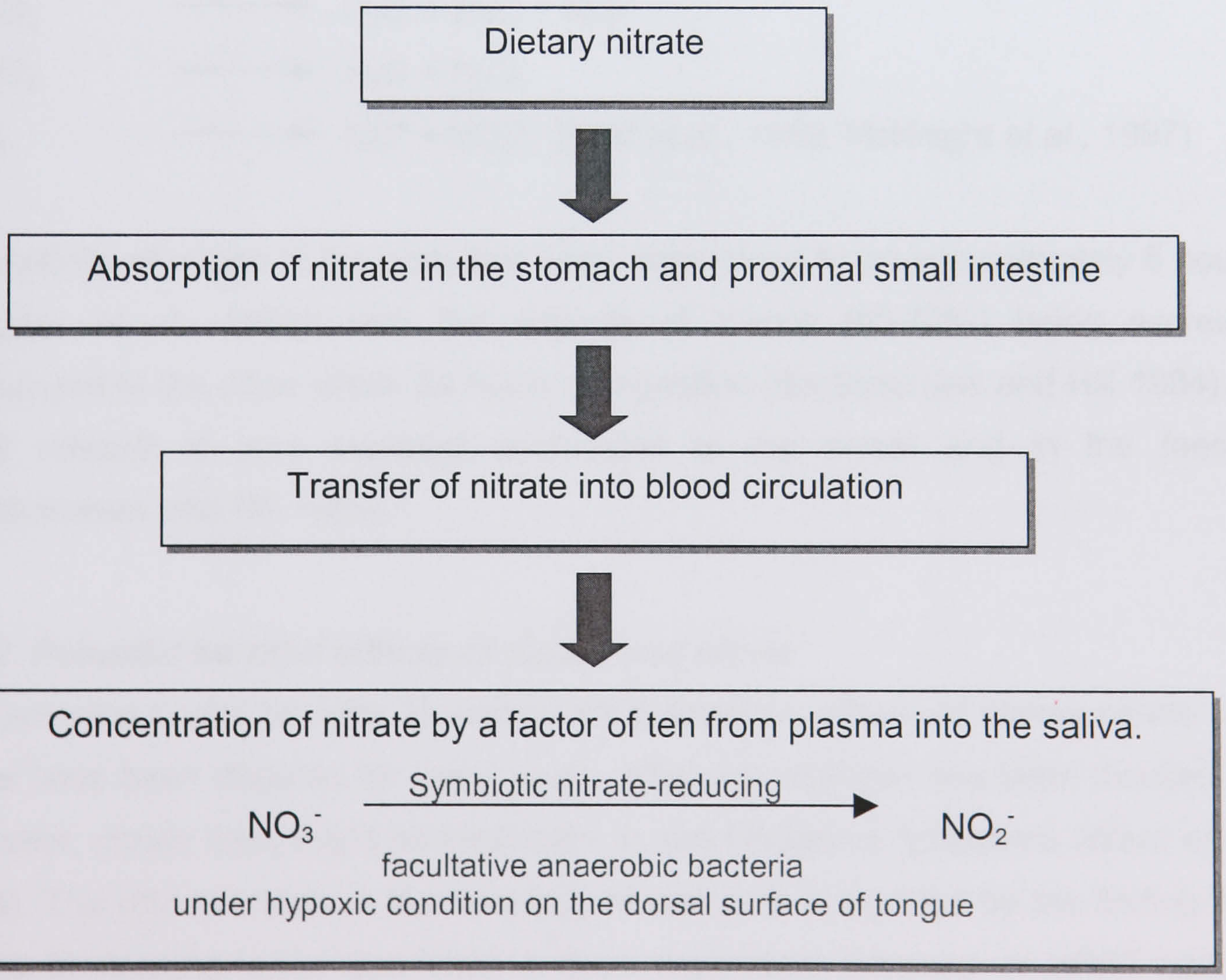


Fig 1.8 A schematic diagram of the enterosalivary circulation of dietary nitrate.

The fate of dietary nitrate and nitrite in the human body is illustrated in figure 1.8. Gastric nitrite is derived from three sources, namely the direct ingestion from cured meats, baked food and preserved food, enterosalivary circulation of nitrate (Fig. 1.8) and finally, its formation in the gastric cavity by *in situ* bacterial reduction (Kyrtopoulos 1989). Exogenously consumed nitrite contributes a relatively small fraction to the total gastric nitrite load. Nitrite formation in the stomach of healthy individual is negligible since bacteria cannot survive in the acidic condition of the stomach (pH of normal gastric juice <3). Although only about 5% of all nitrates entering the mouth is converted to nitrite, this route in fact makes a major contribution (80%) to gastric nitrite load (Kyrtopoulos 1989).

From the salivary and dietary nitrate, a high concentration of nitrite is generated in the mouth. When swallowed into the acidic environment of the stomach, nitrite is rapidly protonated to form nitrous acid (HNO_2), which in turns decomposes to various reactive nitrogen species spontaneously:

$NO_2^- + H^+ \longrightarrow HNO_2$ (pKa 3.2 – 3.4)

$HNO_2 + H^+ \longrightarrow H_2NO_2 \longrightarrow H_2O + NO^+$

$3HNO_2 \longrightarrow H_2O + 2NO + NO_3^-$

$2HNO_2 \longrightarrow H_2O + N_2O_3$

$N_2O_3 \longrightarrow NO^\bullet + NO_2^\bullet$ (Leaf *et al.*, 1989, McKnight *et al.*, 1997)

The half-life of nitrate in the body has been determined to be approximately 5 hours (Wagner *et al.*, 1983), with the majority of nitrate (60-70%) being excreted unchanged in the urine within 24 hours of ingestion (Bartholomew and Hill 1984). A small amount is also excreted unchanged in the sweat and in the faeces (Bartholomew and Hill 1984).

1.3.2 Potential harmful effects of nitrate and nitrite

The potential health benefits as well as the deleterious effects of dietary nitrate and nitrite have been debated for many years. Although attention has been focused on the latter, nitrate itself might be implicated in non-Hodgkin's lymphoma (Ward *et al.*, 1996). The role of nitrate in the aetiology of cancer is supported by the finding that nitrate in drinking water can elicit a dose dependent increase in HPRT variant frequency in peripheral lymphocytes. This is a marker of DNA damage, and an increase in its level is a prerequisite for tumour formation (van Maanen *et al.*, 1996).

Dietary nitrate may be associated with gastric cancer via its conversion to nitrite by the oral microflora. It is known that nitrous acid formation in the stomach may lead to production of nitroso-compounds. These compounds are capable of nitrosating secondary amines ingested in food, resulting in the production of carcinogenic nitrosamines (Sen *et al.,* 1969). Ingestion of nitrite has also been shown to be positively associated with p53 mutations, a gene/protein that suppresses the growth of tumours (Palli *et al.,* 1997).

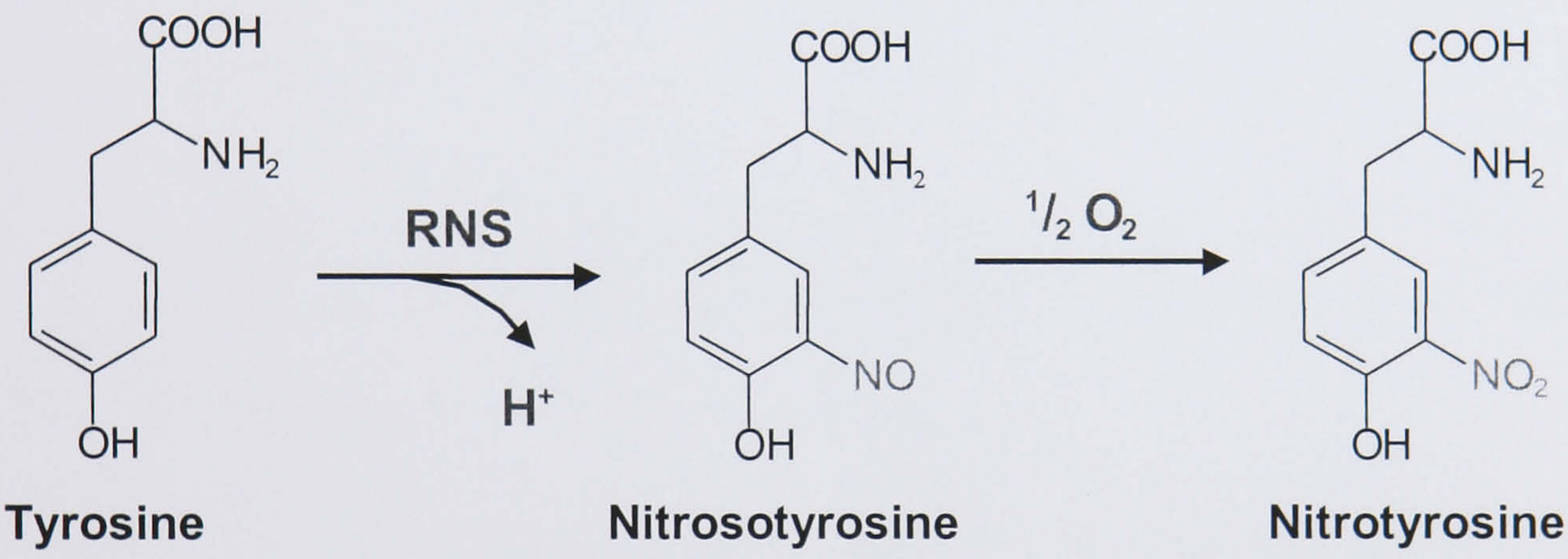
In spite of most studies concentrating on the potential of nitrate as a harmful dietary component, other studies have suggested a role of dietary nitrate in host defence. Helicobacter pylori are the most common bacterial pathogen worldwide, causing chronic active gastritis as well as being associated with duodenal and gastric ulcer (Graham *et al.,* 1992, Parsonnet 1993). Addition of nitrite to acid (0.2 M HCl/KCl buffer, pH 2) (in concentrations found in the saliva after a nitrate-rich meal) resulted in complete killing of the bacteria while acid alone allowed growth to continue (Dykhuizen *et al.,* 1998, Dykhuizen *et al.,* 1996). Nitrate can also mediate beneficial effects to the human body via production of nitric oxide in the stomach, thereby inhibiting platelet aggregation (McKnight *et al.,* 1999).

1.3.3 Reactive nitrogen species and tyrosine nitration

Reactive nitrogen species is a collective term to describe highly reactive species derived from nitrogen. These include not only radicals (nitric oxide, $NO^{\bullet}$ and nitrogen dioxide radical, $NO_2^{\bullet}$), but also non-radicals (nitrous acid, HNO_2 and peroxynitrite, $ONOO^-$). Reactive nitrogen species have been implicated in the pathogenesis of many diseases such as atherosclerosis, gastritis and neurodegenerative diseases (Beckman 1994, Mannick *et al.,* 1996, Smith *et al.,* 1997, Good *et al.,* 1998).

Nitration of tyrosine in proteins is one of the mechanisms whereby RNS contributes to the pathogenesis of diseases. Steady state concentration of free 3-nitrotyrosine in human plasma has been estimated to be about 0.05% of that observed for tyrosine (Kamisaki *et al.,* 1996). However, in pathological conditions, a 2-10 fold increase in the magnitude of protein 3-nitrotyrosine and a 1.5-2 fold increase in the nitration of free tyrosine has been reported depending on the disease and tissue (Greenacre and Ischiropoulos 2001). For instance, widespread occurrence of nitrotyrosine has been detected in neurons in brain tissues from cases of Alzheimer's disease (Smith

et al., 1997), Parkinson's disease (Good *et al.,* 1998), human atherosclerotic lesions (Beckman 1994), myocardial inflammation (Kooy *et al.,* 1997), gastric cancer (Goto *et al.,* 1999), lung cancer (Pignatelli *et al.,* 2001) and in lung tissue from patients with adult respiratory distress syndrome (Kooy *et al.,* 1995). Reactive nitrogen species such as peroxynitrite (Ischiropoulos *et al.,* 1992, Kooy and Royall 1994, Carreras *et al.,* 1994), nitrogen dioxide (Oury *et al.,* 1995, Kikugawa *et al.,* 1999) and those produced from acidic nitrite (Oldreive *et al.,* 1998, Knowles *et al.,* 1974) may occur *in vivo* and are able to nitrate both free and protein-bound tyrosine. Nitration of tyrosine, mediated by nitrite under acidic conditions, has been proposed to proceed via a two-step mechanism (figure 1.9), involving an initial nitrosation step and the subsequent oxidation of the nitroso group to yield 3-nitrotyrosine (Knowles *et al.,* 1974).



Tyrosine **Nitrosotyrosine** **Nitrotyrosine**

Fig 1.9 The mechanism of tyrosine nitration by RNS derived from nitrite in acid.

Nitration of protein-bound tyrosine residues induces a change in polarity that may alter protein/enzyme activity. Several examples of the implication of tyrosine nitration *in vivo* are listed below:

- Altered cell morphology due to disrupted microtubule organisation (Eiserich *et al.,* 1999).
- Reduced antioxidant defence resulting from diminished activity of superoxide dismutase (MacMillan-Crow and Thompson 1999).
- Impaired cellular function due to reduced GTP binding (Rohn *et al.,* 1999).
- Interference with the signalling cascade as a result of inactivation of tyrosine kinases (Gow *et al.,* 1996, Crow *et al.,* 1997).

It has been demonstrated that dietary nitrate and nitrite can contribute to the formation of 3-nitrotyrosine in the body under acidic conditions, such as those found in the stomach, through the formation of RNS (Oldreive *et al.,* 1998, Knowles *et al.,* 1974). The absorption of orally administered 3-nitrotyrosine and the presence of its metabolites 3-nitro-4-hydroxyphenylacetic acid (NHPA) and 3-nitro-4-hydroxyphenyllactic acid (NHPL) in the urine have been demonstrated previously in rats (Ohshima *et al.,* 1990). However, α-tubulin remains as the only protein that has been shown to be capable of incorporating free 3-nitrotyrosine within cells (Eiserich *et al.,* 1999). Flavonoids such as catechin and epicatechin were demonstrated to inhibit acidic nitrite-mediated tyrosine nitration (Oldreive *et al.,* 1998). Thus, the ability of monomeric and dimeric cocoa procyanidins to inhibit acidic nitrite-mediated tyrosine nitration, and the implications for protective effects in the GI tract was investigated.

1.4 OBJECTIVES

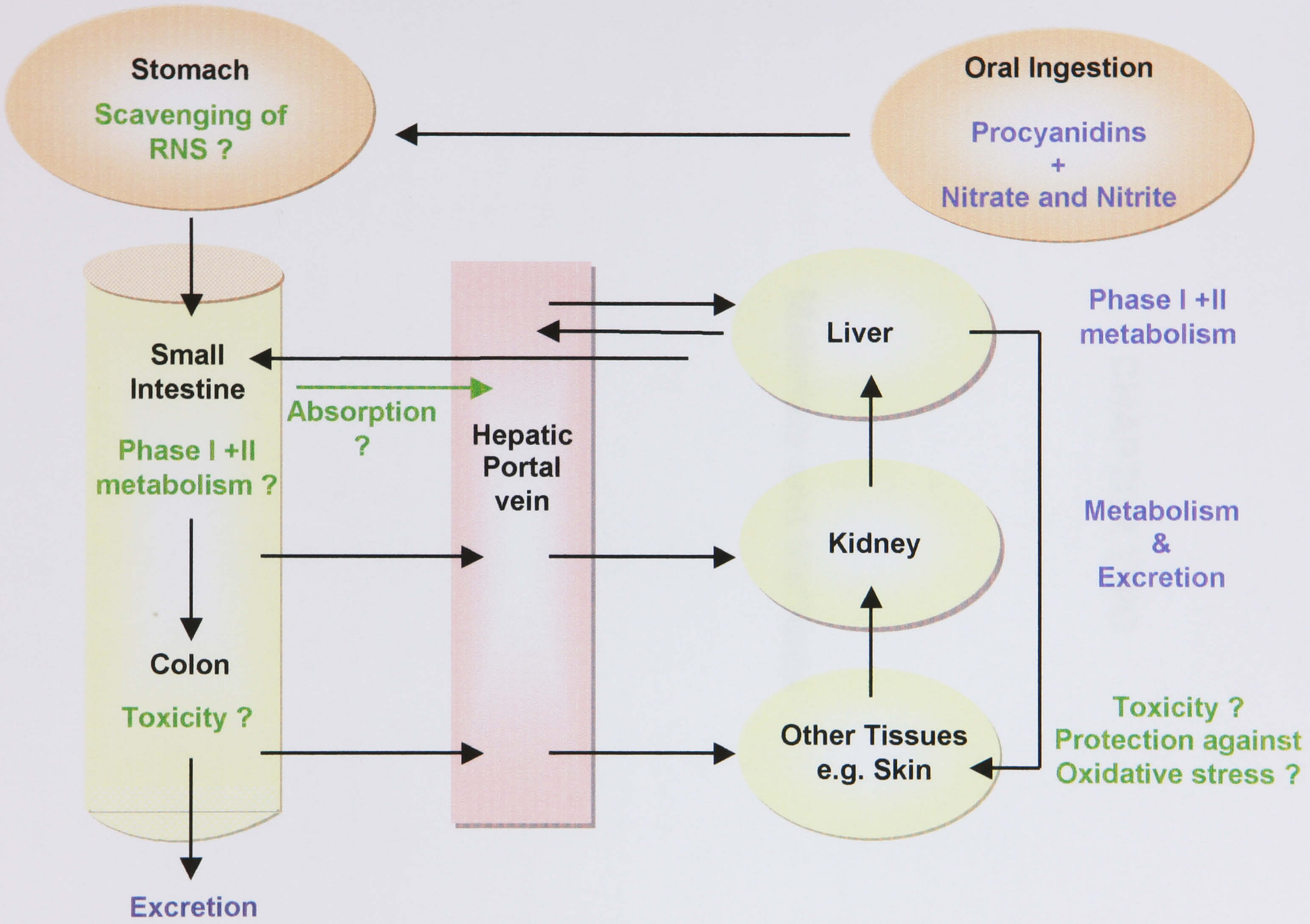
Nitrite under acidic conditions, as in the gastric lumen, contributes to the formation of reactive nitrogen species (Oldreive *et al.,* 1998), potentially leading to alteration of protein and enzyme activities. Recently, there has been considerable interest in the ability of polyphenols to scavenge RNS. Cocoa beans (*Theobroma cacao*) are extremely rich in polyphenols in the form of procyanidin oligomers of epicatechin, comprising 12-18% of the dry weight of the whole bean. The aim of this thesis was to investigate the potential protective effects of flavonoids, in particular monomeric and dimeric procyanidins isolated from cocoa, against RNS and the possible biological significance. Thus the objectives were:

1) To investigate the ability of procyanidins to scavenge RNS derived from acidic nitrite (akin to conditions in the stomach) by examining their ability to inhibit tyrosine nitration.

2) To elucidate the mechanism underlying the antioxidant activity of procyanidins by comparing the reaction profile of other flavonoids with RNS derived from acidic nitrite.

3) To establish the absorption and metabolism of nitrosated products across the small intestine, using human Caco-2 cells and isolated rat jejunum as models of the small intestine.

4) To investigate the biological activities of nitrosated products in terms of cytotoxicity and the ability to protect cells against oxidative stress-induced damage.


Stomach
Scavenging of RNS ?
Oral Ingestion
Procyanidins
+
Nitrate and Nitrite
Small Intestine
Phase I +II metabolism ?
Colon
Toxicity ?
Excretion
Absorption ?
Hepatic Portal vein
Liver
Phase I +II metabolism
Kidney
Metabolism & Excretion
Other Tissues e.g. Skin
Toxicity ? Protection against Oxidative stress ?

CHAPTER TWO

Materials and Methods

Materials and methods

2.1 MATERIALS

All chemicals used in these studies were of the analytical grade purity or tissue culture grade where necessary. All buffers and solutions were prepared using ultrapure water (18.2 MΩ, ELGA, Buckinghamshire, UK). The majority of the chemicals: *p*-Anisaldehyde, basic bismuth nitrate, bovine serum albumin (BSA), (+)-catechin, cyclosporin A, diphenylboric acid 2-aminoethyl ester, DMSO, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, (-)-epicatechin, *o*-dianisidine, GF120918, glucose, glucose oxidase, β-glucuronidase, 4-hydroxy,3-nitrophenylacetic acid, [^{14}C]-mannitol, 4-methylumbeiliferon, 4-methylumbeiliferon glucuronide, 3-nitrotyrosine, peroxidase, polyethylene glycol 4000, potassium iodide, quercetin, rhodamine 123, simulated gastric juice (without pepsin) (pH 1.5), sodium nitrite ($NaNO_2$), tartaric acid, tyrosine, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), verapamil and the colourimetric Sulforhodamine B assay kit were obtained from Sigma-Aldrich Chemical Company (Dorset, UK). The polyphenolic compounds chrysin, cyanidin-3-rutinoside, epicatechin gallate, epigallocatechin gallate, hesperedin, hesperetin, kaempferol, naringenin, naringenin-7-glucoside, pelargonidin-3-glucoside, procyanidin dimer B2, resveratrol, rosmarinic acid were purchased from Extrasynthese (Genay, France). Epicatechin dimer purified from Cocoapro cocoa [23, 24] was supplied by Mars Incorporated, Hackettstown, New Jersey, USA. PSC833 (Valspodar) was a kind gift from Dr. M. Lemaire (Novartis, Basle, Switzerland).

Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (heat inactivated), Hanks' balanced salt solutions (HBSS), L-glutamine, Minimum essential medium (MEM), non essential amino acids, penicillin-streptomycin solution, phosphate-buffered saline (PBS, sodium bicarbonate, trypsin-ethylene diamine tetraacetic acid (EDTA) were obtained from Life Technologies Ltd. (Paisley, UK). Foetal calf serum 'GOLD' was purchased from PAA Laboratories (Somerset, UK).

AnalaR grade ethyl acetate, methanol, acetonitrile, acetic acid, formic acid, hydrochloric acid and toluene for thin layer chromatography (TLC) were obtained from Merck (Dorset, UK). HPLC grade methanol and acetonitrile were obtained from Rathburn Chemical Ltd. (Walkerburn, Scotland). All other reagents used were of analytical grade.

Materials and methods

Silica gel 60 F_{254} sheets and C_{18} octadecyl sheets were purchase from Merck Ltd., Lutterworth, Leicester.

A Nova-Pak C_{18} column (250 mm x 4.6 mm) with a 4 μm particle size was purchased from Waters (Watford, UK). The Luna C_{18} column (250 mm x 10 mm) with a 5 μM particle size used for reverse phase preparatory HPLC was obtained from Phenomenex (Cheshire, UK).

2.2 METHODS

2.2.1 Interaction of procyanidins with acidic nitrite-generated reactive nitrogen species

2.2.1.1 Interaction of tyrosine with acidic nitrite

3-Nitrotyrosine formed in the presence of acidic nitrite was identified using reverse phase HPLC. Stock solutions of 1 mM tyrosine and sodium nitrite ($NaNO_2$) were prepared in 18.2 MΩ water. Tyrosine (400 μM) was incubated with $NaNO_2$ (400 μM) at 37°C for 4h (Oldreive *et al.,* 1998), and the reaction was terminated by placing the reaction mixtures on ice prior to HPLC analysis (see below). 3-Nitrotyrosine was identified by comparison of the UV spectra of samples and by spiking with an authentic standard. Standard solutions of tyrosine (0, 40, 80, 120, 160 and 200 μM) and 3-nitrotyrosine (0, 2.5, 5.0, 7.5, 10 and 15 μM) were run using the same HPLC protocol to construct calibration curves.

2.2.1.2 HPLC Analysis

Reverse phase HPLC was carried out using a Hewlett Packard 1100 HPLC system with an auto-injector, auto-sampler, and diode array detector linked to a HP ChemStation for data collection and processing. The sample was injected onto a Nova-Pak C_{18} (250 mm x 4.6 mm i.d., 4 μm) column using an autoinjector. The mobile phase A consisted of methanol/water/5N HCl (20/79.1/0.1 v/v/v) and mobile phase B of 100% acetonitrile. The following gradient system was used (min/% acetonitrile): 0/5, 30/50, 35/50, 36/5, 40/5 with a flow rate of 0.5 ml/min. The eluent was monitored using photodiode array detection (DAD) set at 280 nm with product spectra obtained between 220-600 nm. 4-Hydroxy-3-nitrophenyl-acetic acid (NHPA, 50 μM) was added to all reaction mixtures prior to HPLC analysis as internal

standards. 4-Hydroxy-3-nitrophenyl-acetic acid was chosen because it is a breakdown product of tyrosine by the human body and absorbed at similar wavelength (280 nm) as 3-nitrotyrosine.

2.2.1.3 Inhibition of tyrosine nitration by procyanidins

The abilities of catechin and epicatechin dimer to inhibit acidic nitrite-mediated tyrosine nitration were studied. Stock solutions of 1 mM catechin monomer and epicatechin dimer were prepared in 18.2 MΩ water. Equimolar concentration (400 μM) of tyrosine and $NaNO_2$ were co-incubated with flavonoids (50 to 400 μM) in 0.5 M HCl at 37°C for 4h (Oldreive *et al.,* 1998). Reactions were terminated by placing the reaction mixtures on ice prior to HPLC analysis.

2.2.1.4 Direct interaction of procyanidins and flavonoids with acidic nitrite

Stock solutions of 1 mM $NaNO_2$, catechin and epicatechin monomer, epicatechin dimer were prepared in 18.2 MΩ water. Stock solutions of 10 mM hesperetin and methylated epicatechin were prepared in methanol. Sodium nitrite, $NaNO_2$ (400 μM) and flavonoids (50 to 400 μM) were incubated in 0.5 M HCl for 2h at 37°C. The reactions were terminated by placing the reaction mixtures on ice prior to HPLC analysis.

2.2.1.5 Preparative HPLC

In order to identify the products derived from interaction of catechin monomer and epicatechin dimer with acidic nitrite, preparative HPLC was applied to isolate and collect the individual components for further structural characterisation using LC-MS/MS. Equimolar concentration (400 μM) of catechin monomer/epicatechin dimer and $NaNO_2$ were incubated in simulated gastric juice for 2h at 37°C. The reactions were terminated by placing the reaction mixtures on ice. Samples were then freeze-dried overnight and reconstituted in simulated gastric juice prior to isolation of products by preparative HPLC.

Individual products were separated by reverse phase HPLC using a Luna C_{18} (250 mm x 10 mm i.d., 5 μm) column. The mobile phase A consisted of methanol/water/5N HCl (20/79.1/0.1 v/v/v) and mobile phase B of 100% acetonitrile. The following gradient system was used (min/% acetonitrile): 0/5, 30/50, 35/50,

36/5, 40/5 with a flow rate of 2 ml/min. The eluent was monitored at 280 nm with spectra of products obtained over the 220-600 nm range.

For interactions of epicatechin monomer, methylated epicatechin and hesperetin with acidic nitrite, the reaction products were directly identified by LC-MS/MS analysis.

2.2.1.6 Mass spectrometry

Mass spectrometry was developed at the beginning of the 20th century (Thomson 1913), mainly used as a technique by physicists to determine the structure of atoms. Its used was spread to the identification and quantitation of organic samples in the 1940s. However, prior to the mid-1980s to the early 1990s, mass spectrometry was only applicable to samples that existed in, or could be put into the gas phase as neutral molecules before ionisation could occur. This is due to the limitations in the ionisation process available at the time. Since then, various desorption/ionisation techniques have been developed, which enable the production of gas phase ions from samples in solution or in a solid matrix for separation according to their *m/z* values. This has opened up a new dimension of research in the field of biological and life sciences.

Mass spectrometry is an analytical technique used frequently to identify unknown compounds, quantify known materials and elucidate the structural and physical properties of ions. The basis of mass spectrometry is the separation and detection of gas-phase ions according to their mass as a function of the number of charge states (mass-to-charge ration –*m/z*) of these ions. It is a technique with very high levels of specificity and sensitivity, enabling analysis with minute quantities, e.g. zeptomole (10^{-21} mol) level of neuropeptides (Andren 1994). Structural information can also be generated using certain type of instruments, usually tandem mass spectrometers by fragmenting the sample and analysing the resulting products. The use of mass spectrometry is now routinely applied to the analysis of proteins, peptides, and oligonucleotides, in drug discovery, pharmacokinetics and drug metabolism research as well as in neonatal screening and drug testing.

The mass spectrometer

All mass spectrometers are essentially composed of three parts:

- an ionisation chamber,
- a mass analyser,

Materials and methods

- a detector.

The first step of mass spectrometry is to produce ions in the gas phase, because ions are easier to manipulate than neutral molecules. Compounds are converted into gas phase molecules either before or during the charging or ionisation process. These ions are accelerated to a specific velocity using electric fields, and projected into a suitable mass analyser where they are separated according to their mass-to-charge ratio. When the ions strike the detector, they are converted into an electrical signal, which is sent to a data system where the m/z ratios are stored together with their relative abundance for presentation in the format of a m/z spectrum (Fig 2.1) (Wilson and Walker, practical biochemistry).

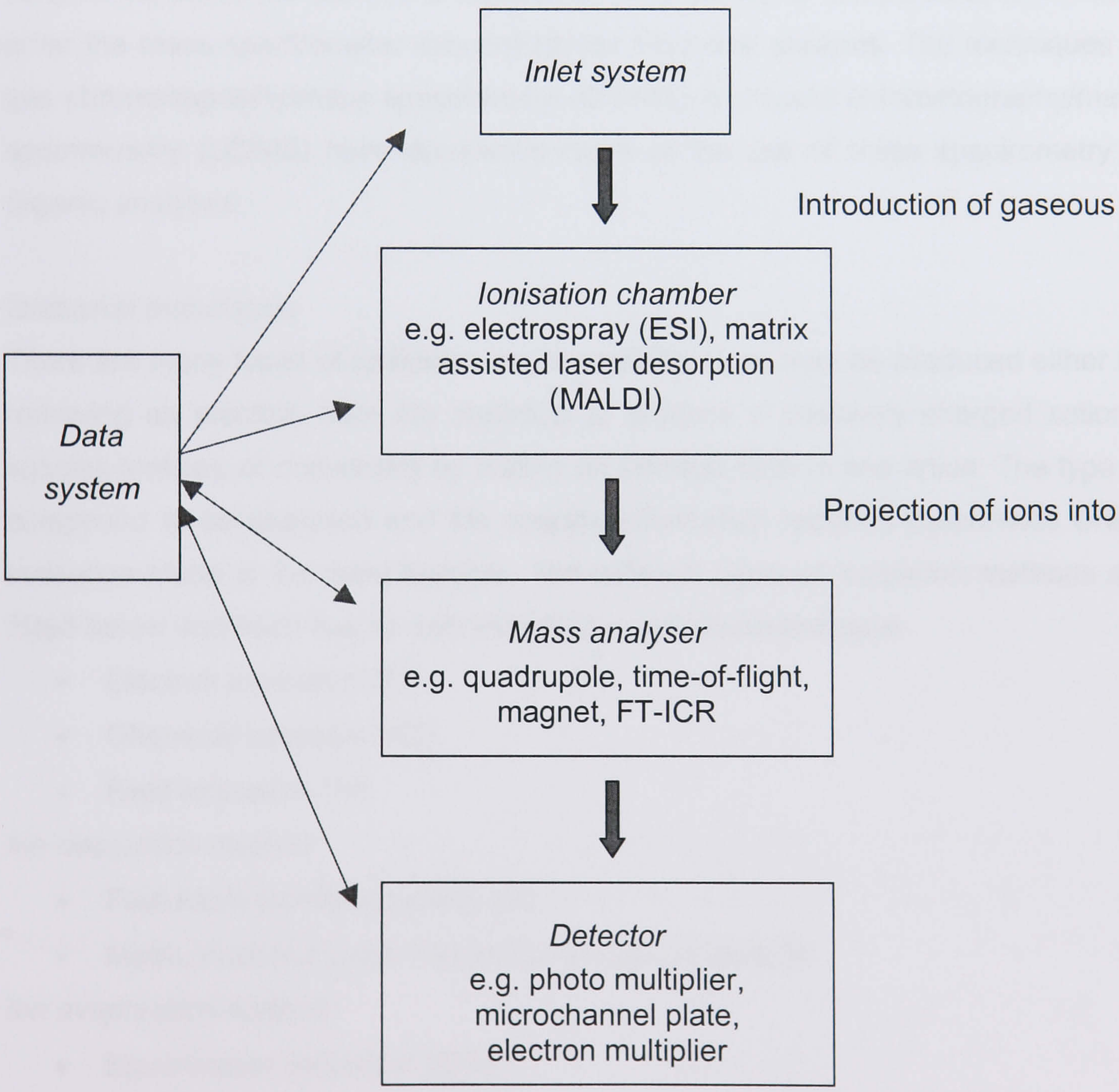


Fig 2.1 The basic principle of a mass spectrometer.

Materials and methods

The whole instrument including the ionisation chamber is maintained under high vacuum (<10^{-5} mBar) to avoid any hindrance from air molecules in the path of the ions when travelling from one end of the instrument to the other. On modern mass spectrometers the entire operation of the instrument, and often the sample introduction can be easily controlled under complete data system.

Sample introduction

There are different methods of sample introduction, including injection of samples directly into the ionisation source or the involvement of chromatography *en route* to the ionisation source. This latter method of sample introduction usually involves the mass spectrometer being coupled directly to a high pressure liquid chromatography (HPLC), gas chromatography (GC) or capillary electrophoresis (CE) separation column. As such, the sample is separated into a series of components which then enter the mass spectrometer sequentially for individual analysis. The techniques of gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS) have dominated much of the use of mass spectrometry in organic analyses.

Ionisation techniques

There are many types of ionisation mode available. Ions may be produced either by removing an electron from the molecule to produce a positively charged cationic species (cation), or conversely by adding an electron to form and anion. The type of compound to be analysed and the specific information required determines which ionisation mode is the most suitable. The different types of ionisation methods are listed below and each has its own advantages and disadvantages:

- Electron Ionisation (EI)
- Chemical Ionisation (CI)
- Field Ionisation (FI)

Ion desorption method

- Fast Atom Bombardment (FAB)
- Matrix Assisted Laser Desorption Ionisation (MALDI)

Ion evaporation method

- Electrospray Ionisation (ESI)

Electron Ionisation is by far the most widely used ionisation technique for analytes that can be put into the gas phase. However, the majority of biochemical analyses

are carried out using ESI and MALDI. It is possible to produce both positively and negatively charged sample ions with most ionisation techniques, but simultaneous detection of both type of ions is obviously not possible. Only one kind of ion may be accelerated out of the source region at any time, despite the fact that both types of ion may have been produced. Cations will be accelerated in either an increasing negative gradient field (attracted towards a negative electrode) or a decreasing positive gradient field (repelled from a positive electrode), and vice-versa for anions. The user must therefore decide whether to detect the positively or negatively charged ions before embarking on an analysis.

A. Electron Ionisation

Electron Ionisation is the original ionisation technique and is still very much used in many biological problems involving metabolic studies, drug studies, pollutants etc.

The principle of EI is relatively simple. A beam of energetic electrons is created inside a heated chamber by passing current through a filament. Many metals, when heated to a sufficiently high temperature of approximately 2000K, lose electrons by diffusion from their surface. Rhenium and tungsten are two metals which are particularly useful because they can be readily drawn into thin filaments. Electrons are removed from the surface of the filament when heated, and gain an energy directly related to the potential applied. Commonly a 70 V potential is applied and the resultant beam would contain 70 eV electrons. The electrons are then attracted away from the filament towards a plate called the trap, which is maintained at a relative positive potential. Samples molecules in the gas phase are directed into this high energy beam, and direct interactions take place, resulting in either loss of an electron from the sample molecule (to product a cation) or electron capture (to produce an anion). A schematic diagram of the FAB process is shown in Fig 2.2.

The major limitation of EI is that the sample molecules to be analysed must be in the vapour state, which limits the applicability to biological materials. On the other hand, the advantages of EI include the vast number of validated EI spectra available in commercial databases and the high degree of fragmentation that the molecular ion undergoes, which results in a great deal of structural information. Electron Ionisation spectra are generally considered to be so reproducible that they can be used for library matching i.e. fingerprinting. These advantages make identifications of unknown compounds easier. Electron Ionisation is such an energetic process, that

in some cases, there is often no molecular ion peak present in the resulting mass spectrum.

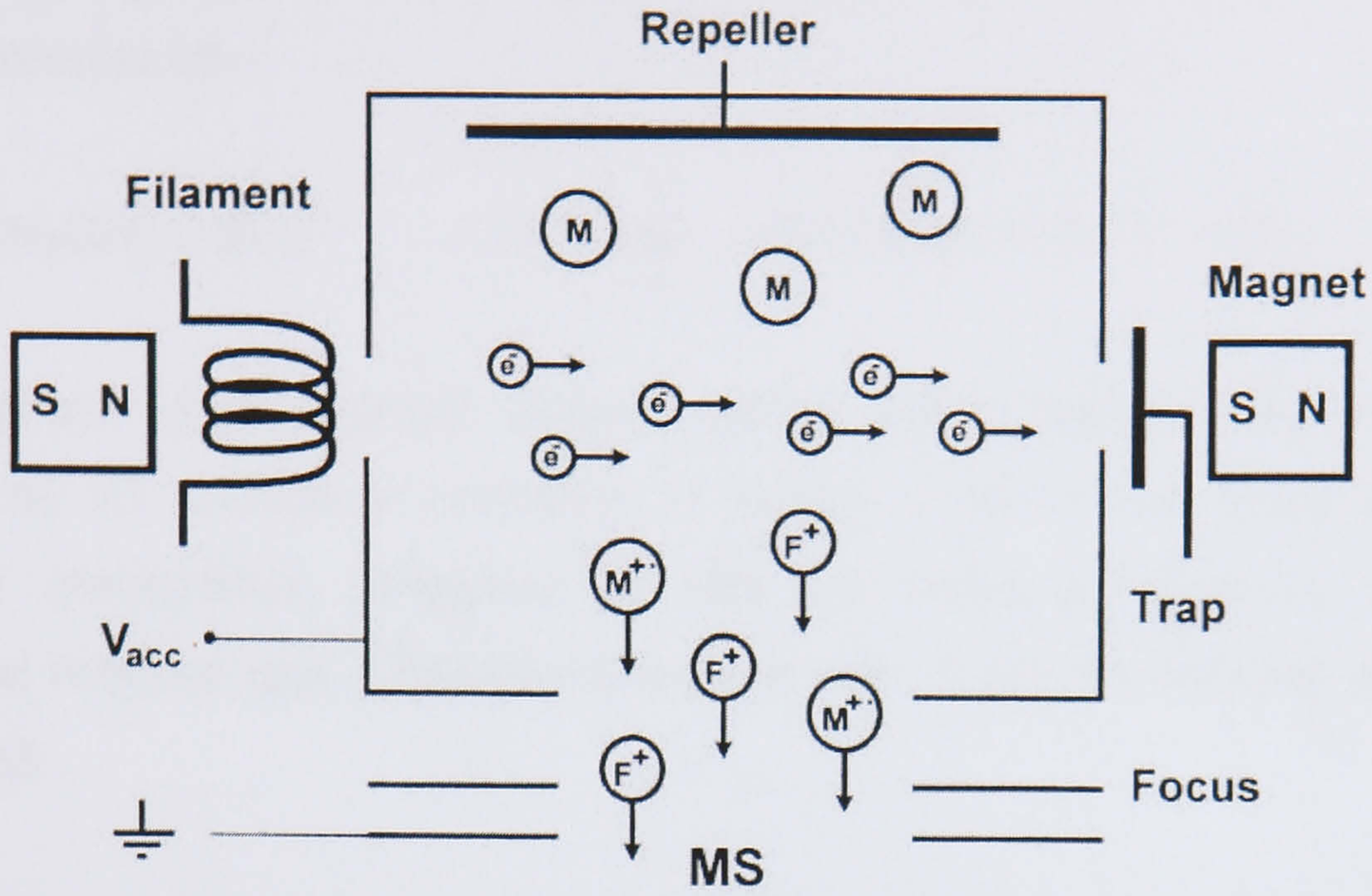


Fig 2.2 Schematic of an Electron Ionisation ion source.

B. Chemical Ionisation

Chemical Ionisation is very similar to the EI source, but little fragmentation occurs giving rise to much cleaner spectra. The use of CI is particularly valuable in the determination of molecular masses, as high intensity molecular or pseudomolecular ions are produced.

The construction of the source is essentially the same as EI, but the beam of electrons is used to create a plasma of ionised reagent gas. This is achieved by filling the source with a suitable reagent gas such as methane (CH_4) or ammonia (NH_3) prior to the analytical experiment. Interaction between the reagent gas and electrons produced by normal EI will give rise to species such as $CH_4^{\neg\bullet+}$ or $NH_3^{\neg\bullet+}$. However, owing to the relatively high pressure of the reagent gases in the source, the possibility of ion-molecule reactions arises. For example:

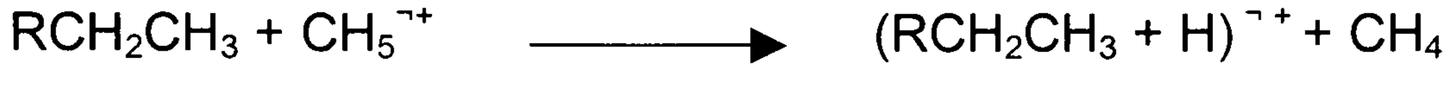
or:

$$CH_4^{\neg\bullet+} + CH_4 \longrightarrow CH_5^{\neg+} + CH_3^{\bullet}$$

$$NH_3^{\neg\bullet+} + NH_3 \longrightarrow NH_4^{\neg+} + NH_2^{\bullet}$$

The resulting ions ($CH_5^{\neg+}$ and $NH_4^{\neg+}$) are strong proton donors (Lewis acids) in the vapour state. When the sample to be analysed is introduced into the source,

ionisation is the achieved by interaction of the sample molecule with the reagent gas, not by direct interaction with the electron beam. This is a less energetic procedure than EI and the most common ion formation in CI is that of the *protonated molecule*, giving rise to a thermodynamically relatively stable parent plus one pseudomolecular ion:



These ions are often relatively stable, tending not to fragment as readily as ions produced by EI. Chemical Ionisation is widely used in the study of drugs and secondary metabolites. Negative CI can be made possible by choosing an appropriate reagent gas. Electron Ionisation and CI are techniques primarily used with GC/MS .

Electron Ionisation and Chemical Ionisation are usually the technique of choice fo small (<800 Da), volatile, thermally stable compounds. However, CI tends to give molecular weight information whereas EI is more useful for structural information.

C. Field Ionisation

This is another method of ionisation requiring the sample to be introduced in the vapour state. In FI the sample is subjected to an intense electric field (10^7 – 10^8 V cm^{-1}), and the outer bonding electrons are subject to large forces. The energetics are sufficient to overcome the ionisation potential, resulting in an electron being removed to generate a molecular radical cation. These ions can then be accelerated out of the source and into the mass analyser in the usual way.

For all the ionisation techniques introduced so far, a required property of the samples is that the solid would have to be sufficiently volatile (at the low pressures used) to evaporate, or more strictly volatilise, prior to ionisation in the electron beam. However, most biological materials either do not possess this property, or are fragile and thermally labile.

To overcome this problem, a number of important desorption methods have been developed that enable solid materials to be introduced into the mass spectrometer. In addition, the ionisation processes usually occur at room temperature or slightly lower temperature. Desorption/ionization has had a great impact on the use of mass spectrometry for organic analyses. Desorption/ionization produces gas phase ions

from thermally labile non-volatile substances such as organic ionic material, proteins and peptides, and other biological compounds like nucleic acids.

D. Fast atom bombardment

The development of FAB ionisation in the 1980s (Barber 1982, Barber 1981) revolutionised the range of compounds analysable by mass spectrometry, and really opened up the field to most areas of biomedical research. Since its invention it has generated massive interest as well as stimulating developments in other ionisation sources appropriate for biological materials.

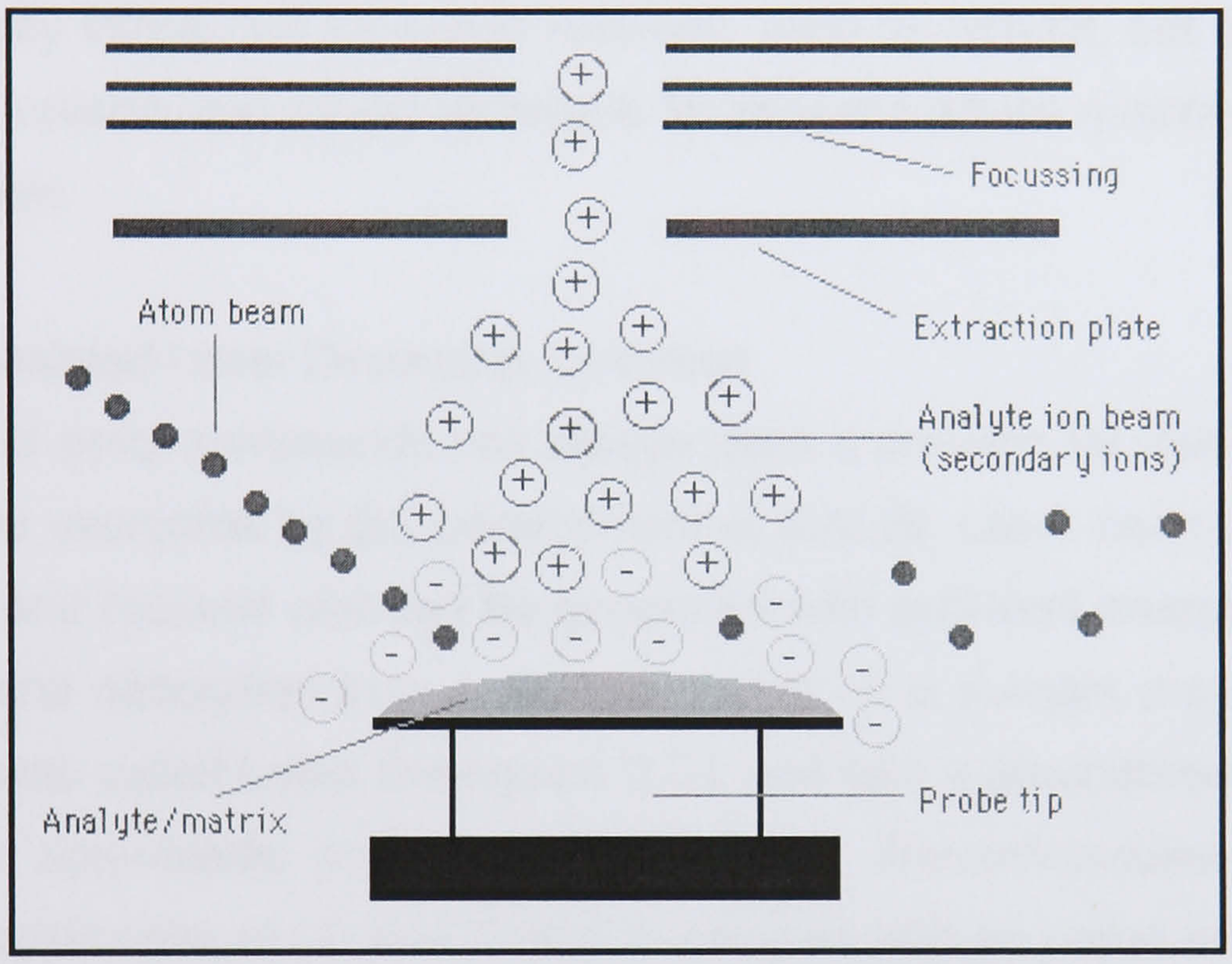


Fig 2.3 Schematic of a Fast Atom Bombardment source (taken from **www-methods.ch.cam.ac.uk/ meth/ms/theory/fab.html**)

The major advantage for the investigation of biological materials is that samples can be introduced into the ionising beam of neutral atoms in solution. The sample is first dissolved in a liquid matrix, which is typically a viscous low vapour pressure liquid such as glycerol, thiolglycerol or 3-nitrobenzyl alcohol. This is used to keep a 'fresh' homogeneous surface for bombardment, thus extending the spectral lifetime and enhancing sensitivity. A few microliters of this liquid are placed on a small metal target at the end of a probe, which is then introduced into the mass spectrometer. Ionisation of the sample takes place by bombardment of the liquid surface with a beam of high kinetic energy atoms (xenon or argon) or ions (caesium). This results in sputtering of molecules from the surface, with the ions of interest almost certainly solvated with matrix molecules. This helps to stabilise the ions by dissipating excess

Materials and methods

energy and limiting fragmentation, giving rise to ions that are usually stable, and exhibit little fragmentation. A schematic diagram of the FAB process is shown in Fig 2.3.

Fast atom bombardment is a comparatively soft ionization technique, typically producing large peaks for the pseudo-molecular ion species $[M+H]^+$ and $[M-H]^-$, along with structurally significant fragment ions and some higher mass cluster ions and dimers. Fast atom bombardment is useful for larger (up to *ca.* 5000 Da), involatile, polar, thermally unstable molecules such as peptides, small proteins and other biopolymers. Nowadays FAB is considered insensitive when compared to more recently introduced ionisation methods, such as MALDI, but it still has a role as a rapid, reliable and robust technique for samples where quantity and purity are not a problem.

E. Matrix Assisted Laser Desorption Ionisation

The study of polar compounds has always been a problem for mass spectrometry, but this was overcame by the development of MALDI. Laser beams can be readily collimated and focused and can be generated with sufficient energy to cause both ionisation and desorption from a sample coated on a suitable probe surface. This technique was called Laser Desorption (LD), and was a groundbreaking step to the analysis of non-volatile polar biological, organic macromolecules and polymers. However, application of LD was limited to samples with an upper mass limit of 5-10 kDa. The mass limit is probably due to the energy required to cause the resonant excitation and successful energy transfer but at the same time not result in decomposition of the thermally labile analyte molecules. In 1987 MALDI was developed in which the matrix functions as an energy sink (i.e. a substance that absorbs the radiation energy at the wavelength of the coherent laser light used), and thus circumvent the restrictive mass limitations of the technique (Karas 1988). It is important to choose a matrix that is a strong absorbance at the laser wavelength and was of low enough mass to be sublimable (Beavis 1989). Suitable matrices include 2,5-dihydroxybenzoic acid and sinapinic acid. The advantage of MALDI compared to FAB is a dramatic increase in both sensitivity and mass range of analysable compounds.

During ionisation, the sample and matrix are mixed together in an organic or aqueous solvent of mutual solubility. Once the solvent is removed, the matrix and analyte molecules co-crystallize onto a stainless steel target. The target is inserted

Materials and methods

into the mass spectrometer and the surface bombarded with a pulsed laser beam that is typically generated by inexpensive nitrogen lasers with a beam wavelength of 337 nm. Molecules are desorbed from the surface and ionise, usually by protonation or deprotonation. A schematic diagram of the MALDI process is shown in Fig 2.4.

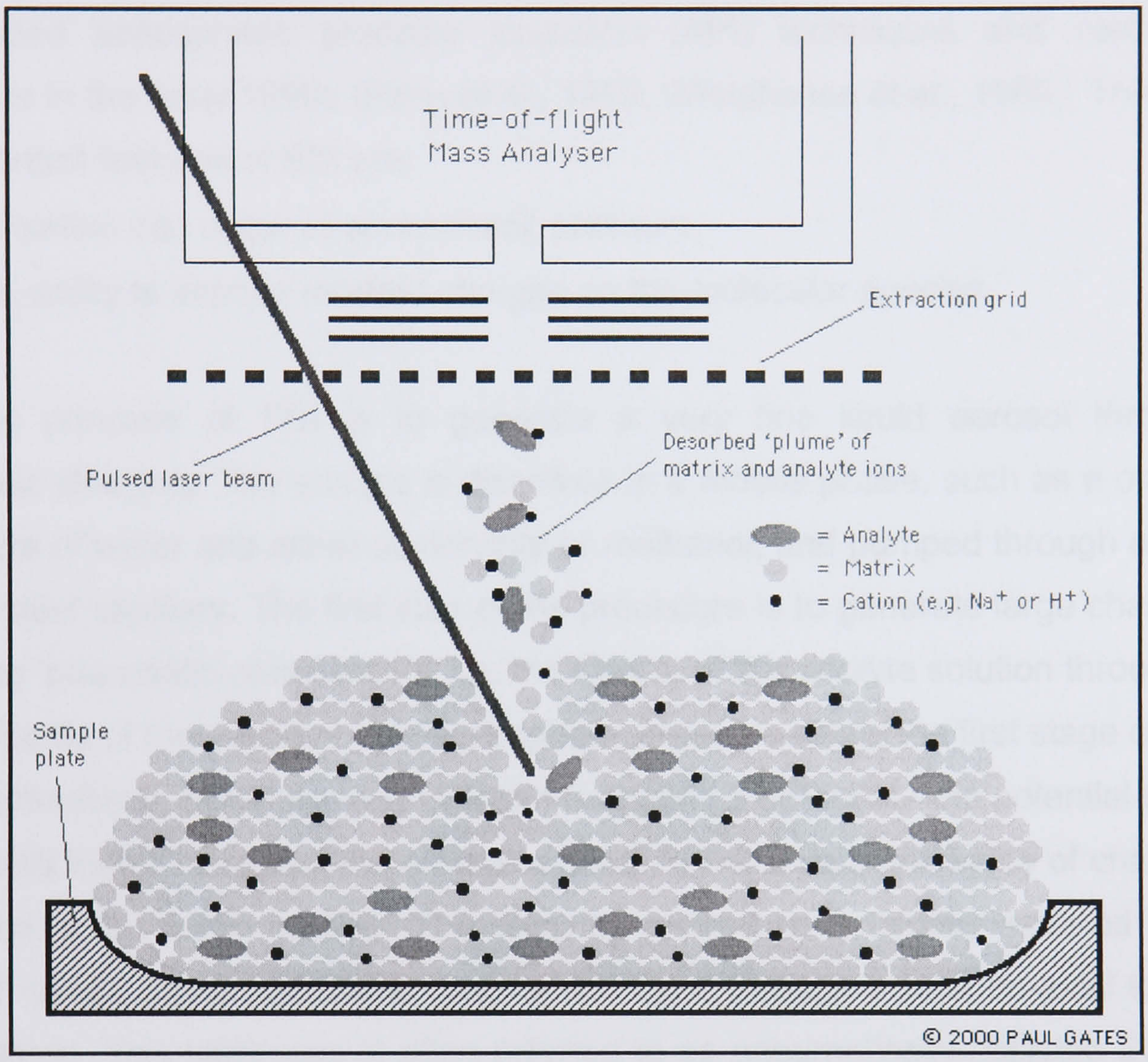


Fig 2.4 Schematic of Matrix Assisted Laser Desorption Ionisation (taken from **www-methods.ch.cam.ac.uk/ meth/ms/theory/fab.html**.

The major advantage of MALDI is the ability to produce large mass ions with high sensitivity, and little fragmentation occurs with the resulting molecular ions (Spengler 1997). This makes MALDI a valuable technique or examining mixtures. The application of MALDI is similar to FAB, but at much higher sensitivity, often 100 – 1000 times better, and has an almost limitless mass range. Proteins with molecular weights above 300,000 Da have been successfully ionised, but because the ions produced are often predominantly singly charged, the large m/z ratio requires the use of time of flight (ToF) mass spectrometers with their virtually unlimited mass range. In addition, because of the short duration of the ion burst following the laser pulse, this technique is unsuitable for scanning analysis on sector or quadrupole

Materials and methods

instruments, but rather particularly suited to ToF mass spectrometry. MALDI is particularly suitable for the ionisation of peptides.

F. Electrospray Ionisation

This technique was developed in the same timeframe as MALDI and with just as great an impact on mass spectrometry and the biological sciences. This is one of the so-called atmospheric pressure ionisation (API) techniques and came to prominence in the early 1990s (Fenn *et al.,* 1989, Whitehouse *et al.,* 1985). The two most important features of ESI are:

- ionisation can occur at atmospheric pressure,
- the ability to impose multiple charges on the molecular species.

The basic principle of ESI is to generate a very fine liquid aerosol through electrostatic charging. The sample is dissolved in a mobile phase, such as a one-to one mixture of water and either acetonitrile or methanol, and pumped through a fine stainless steel capillary. The first step of the procedure is to generate large charged droplets by 'pneumatic nebulisation', i.e. the forcing of the analyte solution through a needle. The tip of the capillary is at atmospheric pressure inside the first stage of the mass spectrometer, but at the end of which is applied a potential - the potential used is sufficiently high to disperse the emerging solution into a very fine spray of charged droplets all at the same polarity. The spraying procedure is usually assisted by a stream of nitrogen gas (nebulising gas), which flows through a tube co-axial to the main capillary. This technique is often referred to as pneumatically assisted ESI or Ionspray (Fig 2.5).

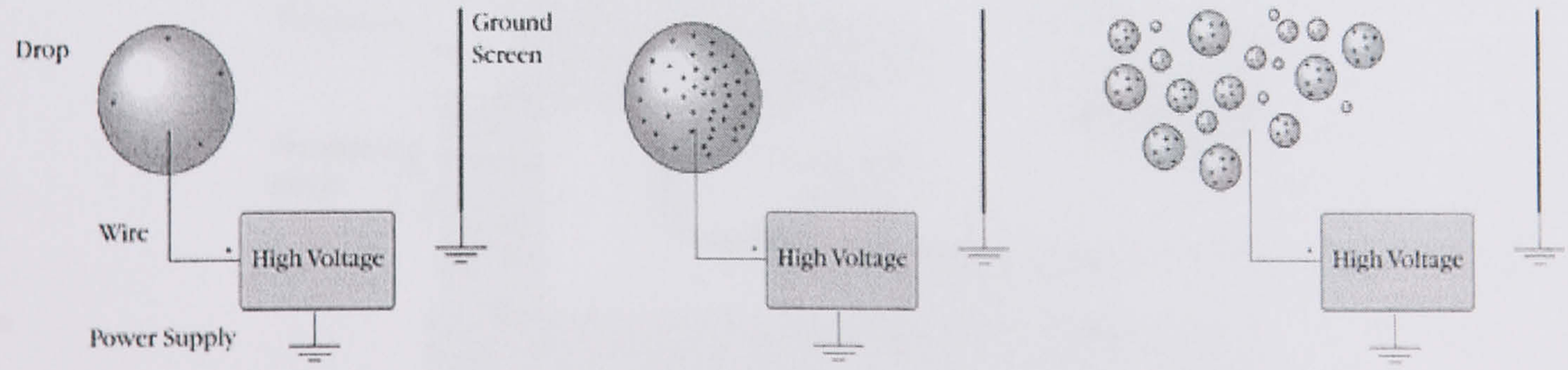


Fig 2.5 A schematic diagram of the formation of multiply charged ions In ESI.

The spray is then directed, via a bath of warm nitrogen gas, through a series of chambers held at increasing vacuum. The solvent evaporates away, shrinking the droplet size and increasing the charge concentration at the droplet's surface. When

the electrical charge reaches a critical state, at the Rayleigh limit, the droplet will violently blow apart again forming a series of smaller, lower charged droplets. The process of shrinking followed by explosion is repeated until individually charged 'naked' analyte (gas phase) ions are formed (Fig 2.5). The final step is to propel the ions towards the high vacuum mass analyser (Gaskell 1997).

Electrospray Ionisation is considered to be one of the softest ionisation techniques available that allows ionisation to occur at atmospheric pressure. As such ESI is the optimum ionisation/vaporisation method for the widest range of polar biomolecules (Griffiths *et al.,* 2001). This has profound significance as it permits for the analysis of non-volatile thermally labile substances. As little energy is transferred to the molecule other than that required for ionisation, even highly polar and thermally labile molecules undergo very little fragmentations. Secondly, owing to the formation of multiple-charge ions from large molecules (>1000–200 Da) in solution, substances that may not be seen in many *m/z* analysers due to the high mass of the substance and the *m/z* limit of the mass spectrometer can be analysed with ESI. This is because the analysis is a function of the mass divided by the number of charge states, i.e. an ion with a mass 100,000 Da and 100 charges can be observed in a mass spectrometer at *m/z* 100. This brings biological macromolecules into the range of many existing mass analysers. Because of these two advantages of ES, this technique has had the greatest impact of any of the various ionization methods on the rapid growth of LC/MS.

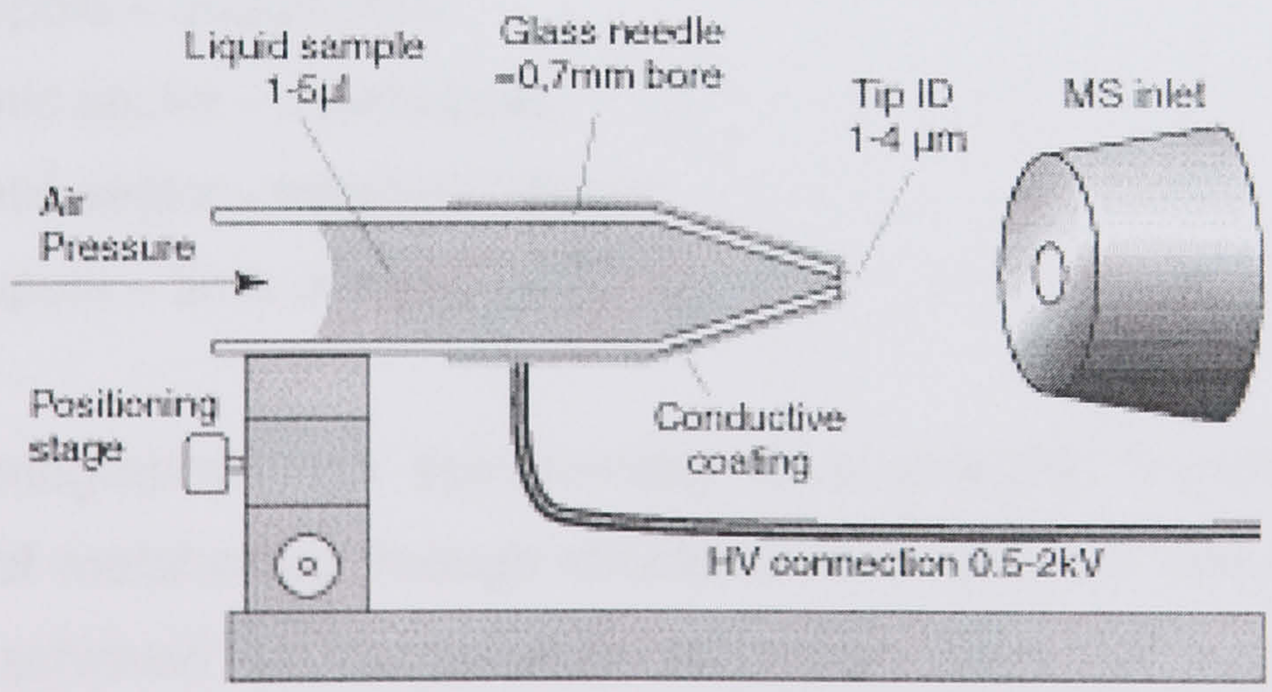


Fig 2.6 A diagram of the most common electrospray apparatus.

Fig 2.6 depicts a diagram of the most common electrospray apparatus used by mass spectrometrists, employing a sharply pointed hollow metal tube, such as a

Materials and methods

syringe needle, with liquid pumped through the tube. A high-voltage power supply is connected to the outlet of the tube and the tube is positioned in front of a plate, (a counter-electrode) that is commonly held at ground potential. When the power supply is turned on and adjusted for the proper voltage, the liquid being pumped through the tube transforms into a fine continuous mist of droplets that fly rapidly toward the counter-electrode.

Tandem mass spectrometry

Apart from Electron Ionisation, all the ionisation techniques described so far are soft ionisation techniques, which primarily produce information about the mass of the intact molecule rather than fragments that can be used to deduce the analyte's structure. When further structural information is desired, fragmentation of the molecule can be obtained by a process called *collisionally activated dissociation* (CAD), also known as *collision-induced dissociation* (CID). This can be achieved by coupling together two analysers, separated by a collision cell. The first analyser is used to select the ion of interest, which is then passed into the collision cell pressurised with an inert gas such as argon. Fragmentation of the original ion, the 'precursor' ion is attained by collision with the atoms in the cell. The dissociated ions are known as 'product' ions, and are then analysed in the second mass spectrometer, generating a product ion mass spectrum of the original precursor ion (de Hoffmann 1996). The analysers can be of the same or of different types. The most common combinations being:

quadrupole – quadrupole

magnetic sector – quadrupole

magnetic sector - magnetic sector

quadrupole – time-of-flight

Liquid chromatography-mass spectrometry is a powerful analytical tool for the identification of metabolites through structural elucidation of potential metabolites. This can be achieved by interpretation of product ion mass spectra of selected precursor ions, guided by concepts of biotransformation pathways (Bu 2000). The basic modes of data acquisition for tandem mass spectrometry experiments are as follows:

A. Full Scan

Materials and methods

The most basic acquisition mode that can be performed is a full ion scan during the complete chromatographic run to look for the original compound and possible metabolites by their calculated mass. However, this is a time consuming method with potentially a large number of possible products to be searched manually. Fortunately, metabolic profiling software such as Metabolite ID, which searches for all possible metabolites, has been developed. A major disadvantage with the full scan is the high signal-to-noise ratio, thus decreasing the sensitivity.

B. Product or daughter ions scanning

This is particularly useful for providing structural information concerning small organic molecules and for peptide sequencing. The first analyser is used to select user specified sample ions, which are pass into the collision cell and bombarded by the gas molecules to cause fragmentation to the ions. These 'product ions' are analysed by the second analyser, and thus producing a fingerprint pattern specific to the compound under investigation.

C. Precursor or parent ion scanning

This is particularly useful for monitoring groups of compounds contained within a mixture that give rise to common product ions under fragmentation, e.g. glucuronide conjugates in urine sample. The first analyser is set to transmit all sample ions, while the second analyser is set to monitor specific fragment ions that are generated by bombardment of the sample ions within the collision cell.

D. Selected or multiple monitoring

This method is used to confirm unambiguously the presence of a compound in a matrix e.g. drug testing with blood or urine samples. As such the compound under investigation must be known and have been well characterised previously. Both of the analysers are static in this method. The first analyser is used to transmit the user-selected specific ions while and the second analyser measures the amount of user-selected specific fragments arising from parent ions. This method is highly specific and highly sensitive.

E. Selected Ion Monitoring (SIM)

This is a more selective recording mode in which the mass spectrometer monitors only the signal for a single m/z ratio, as such provides higher sensitivity over the full scan mode. The SIM mode is only suitable for the kind of instruments in which only the selected ion(s) can reach the detector and trigger a signal.

Materials and methods

F. Selected reaction monitoring (SRM)

SRM is an extended SIM mode using the tandem MS capabilities of the instrument. Similar to SIM, ions of a certain m/z are selected and isolated, but these ions are fragmented, normally by collision-induced dissociation (CID). The resulting product ions formed are monitored with SIM, providing a technique with a very high sensitivity.

G. Neutral Loss Scan

This mode is a speciality of the triple quadrupole instruments and only available with these instruments. The neutral loss scans is particularly useful for investigation of metabolites, e.g. the loss of the glucuronic acid during CID of epicatechin-5-*O*-glucuronide, with epicatechin as detectable ion. In this scan mode, the two quadrupole are set to detect a difference in the m/z ratio corresponding to the anticipated neutral loss. Thus only ions that lose an appropriate 'neutral', for example glucuronic acid, are detected.

The use of tandem mass spectrometry has become an important tool in the structural analysis of flavonoids, and this will be discussed later in this section.

Positive or Negative Ionisation?

Positive ion detection is usually employed if the sample has functional groups that readily accept a proton (H^+). For example amines:

$$R\text{-}NH_2 + H^+ \longrightarrow R\text{-}NH_3^+ \text{ as in proteins and peptides}$$

Negative ion detection is usually employed if the sample has functional groups that readily lose a proton (H^+). For example carboxylic acids:

$$R\text{-}CO_2H \longrightarrow R\text{-}CO_2^+ + H^+, \text{ and}$$

$$R\text{-}OH \longrightarrow R\text{-}O^+ + H^+ \text{ as in saccharides and oligonucleotides}$$

Mass analyser

The main function of the mass analyser is to separate and resolve the ions formed in the ionisation source of the mass spectrometer according to their m/z rations. Different mass analysers have different features, including the m/z range that can be covered, the mass accuracy and the achievable resolution.

Materials and methods

A. Sector analysis

This is considered as the 'classical' method for mass separation and detection. Ions are accelerated from the source, under action of the accelerating voltage, V_a, and enter the electrostatic analyser, which acts as an energy focussing device. The electrostatic analyser focuses ions of the same mass-to-charge ratio into a more coherent path through to the magnetic sector. The magnetic sector consists of a broad flight tube through a variable magnet of field strength and radius. By scanning the field strength all the ions produced in the ion source are sequentially focused at the detector, and the resulting pattern is called a mass spectrum.

B. Time-of-flight

Time-of-flight mass spectrometry (ToF-MS) was developed around fifty years ago, but it is becoming increasing popular along with the introduction of MALDI recently. In ToF instruments the time taken for an ion to travel a set distance is measured very accurately, which is directly related to the m/z ratio of the ion. The major advantages of ToF are that it has a very high sensitivity and a virtually unlimited mass range.

C. Quadrupole mass filters

This type of mass analyser is known as a mass filter and it has the advantage of being a smaller and cheaper device than sector systems, which is particularly useful for hyphenated methods. The disadvantages are lower mass range and sensitivity. In quadrupole mass analyser, only electric fields are used to separate ions according to their m/z ratios. Both direct current (DC) and radiofrequency (RF) voltages are applied to a quadrupole consists of four solid cylindrical rods, through which the ions being separated are passed. Depending on the produced electric field, only ions of a particular m/z will be focused on the detector, while all the other ions will be deflected into the rods. By varying the strengths and frequencies of electric fields, different ions will be detected thus making the mass spectrum (Fig 2.7).

Materials and methods

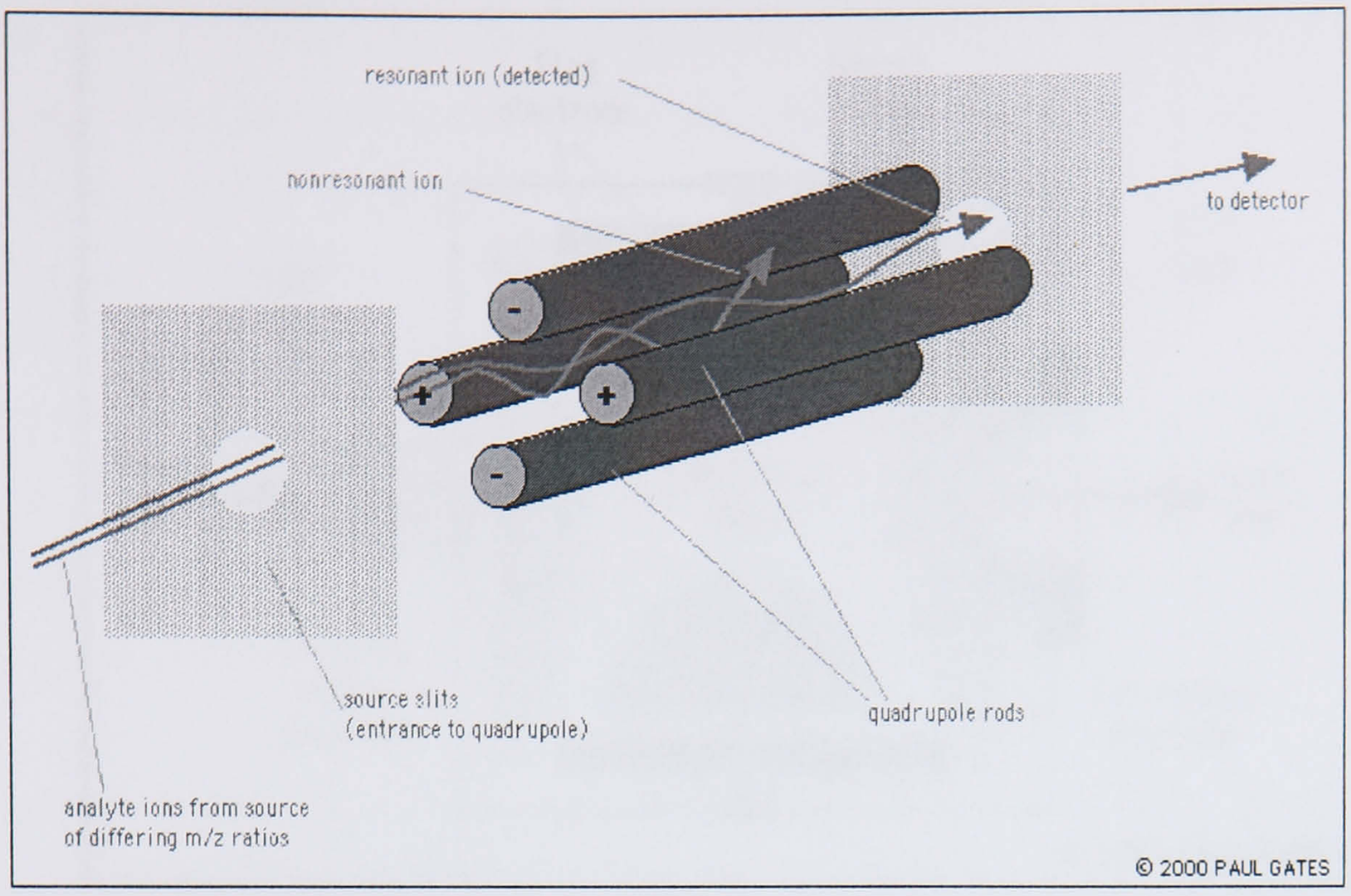


Fig 2.7 A diagram of the ion trap mass analyser (taken from **www-methods.ch.cam.ac.uk/meth/ms/theory/fab.html**.

D. Ion trap

The quadrupole ion trap mass analyser (see figure) consists of three hyperbolic electrodes: the ring electrode, the entrance endcap electrode and the exit endcap electrode. Together these electrodes form a cavity which enables ions to be trapped and analysed (Fig 2.8). The ring electrode is located halfway between the two endcap electrodes and the latters have a small hole in their centres through which the ions can travel. Ions produced from the source enter the trap through the inlet focussing system and the entrance endcap electrode. Various voltages are applied to the electrodes to trap and eject ions according to their m/z ratios. A 3D quadrupolar potential field within the trapping cavity is produced by applying an A.C. potential of constant frequency and variable amplitude to the ring electrode. This will trap ions in a stable oscillating trajectory, which is dependent on the trapping potential and the m/z ratio of the ions. During detection, the electrode system potentials are altered to produce instabilities in the ion trajectories and thus eject the ions in the axial direction. The ions are ejected in order of increasing m/z ratio, focussed by the exit lens and detected by the ion detector system.

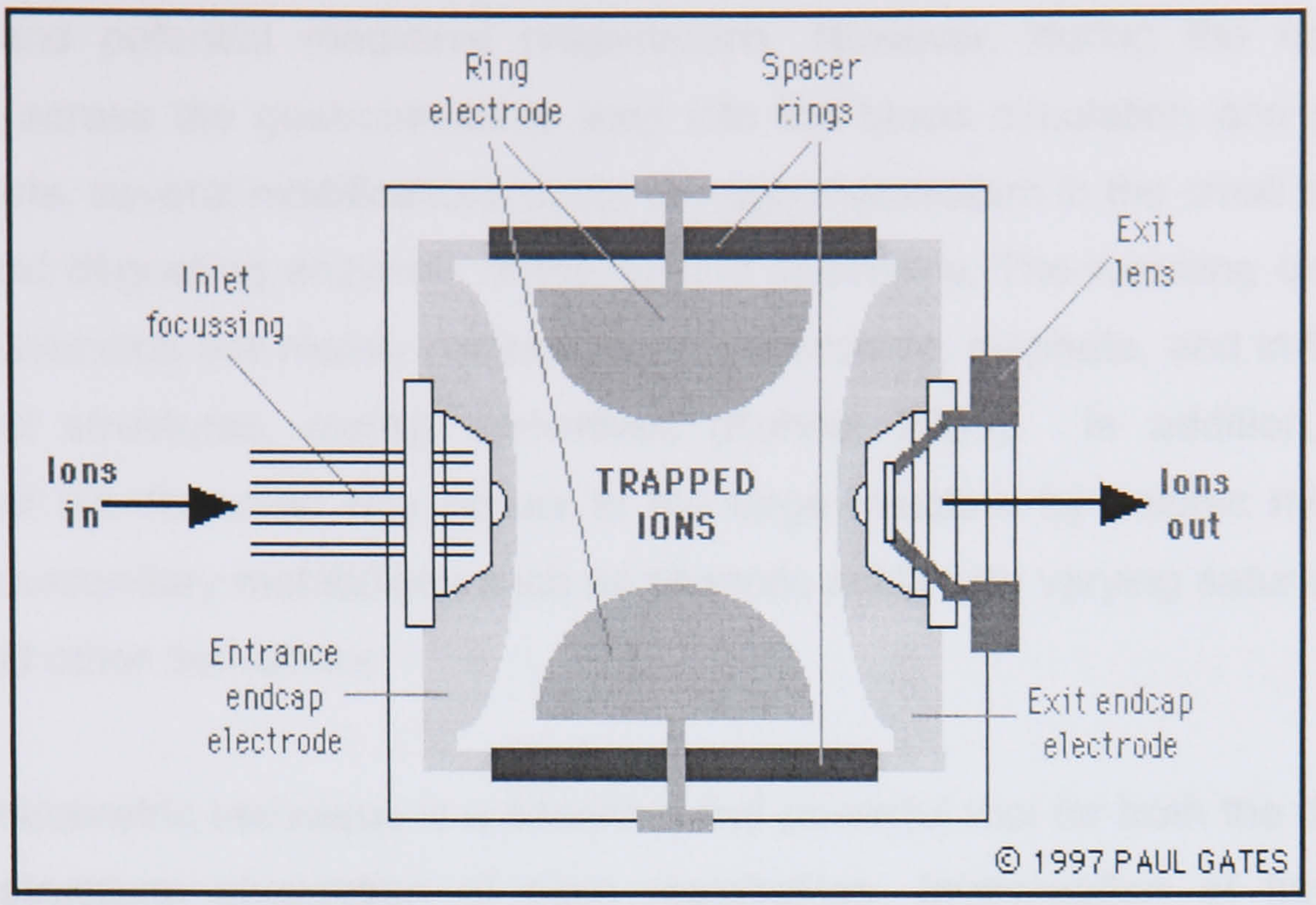


Fig 2.8 A diagram of the ion trap mass analyser (taken from **www-methods.ch.cam.ac.uk/meth/ms/theory/fab.html**.

Detectors

The final component of a mass spectrometer is the detector, which monitors the ion current, amplifies it and the signal is then transmitted to the data system where it is recorded in the form of mass spectra. Most detectors are of the impact or ion collection type. All types of detector require a surface on which the ions impinge and the charge is neutralised either by collection or donation of electrons. This results in electron transfer and an electric current flows that may be amplified and ultimately converted into a signal recorded on a chart or processed by a computer.

For field instruments one sweep of the field strength is defined as a scan. For instances, an electro-magnet scanned from high field to low field will focus ions of high m/z ratio down to ions of low m/z ratio sequentially at the detector. In ToF instruments, ions arrive sequentially at the detector in order of increasing m/z ratio. Different types of detector are available to suit the type of analyser. The more common ones are the photomultiplier, the electron multiplier and the micro-channel plate.

Investigation of flavonoids using mass spectrometry

Most methods developed for the mass spectrometric investigation of flavonoids have focused on biological material from plants due to their importance as natural

Materials and methods

products and potential medicinal preparations. However, during the uptake of flavonoids across the gastrointestinal tract into the blood circulation and potential target organs, several modifications occur through metabolism in the small intestine, the liver and degrading enzymes of the colonic microflora. The resulting circulatory forms of flavonoids are mainly conjugates of glucuronide, sulphate, and in the case of catechol structures, methyl derivatives (Kuhnle 2003). In addition, further cleavage of the flavonoid ring occurs in the large intestine by colonic microflora, producing secondary metabolites such as phenolic acids with varying saturate chain lengths and other derivations.

Mass spectrometric technique is a sensitive and powerful tool for both the detection and the structural elucidation of such metabolites. Investigation of flavonoids, especially regarding fragmentation reactions and structural elucidation, has been undertaken using electron impact and chemical ionisation techniques (Grayer 1989, Kingston 1971, Kingston 1973). This technique is commonly used for the analysis of small and volatile compounds, but it has been possible to yield intense signals for the molecular ion of the flavonoids aglycones (Mabry 1970), which are polar and non-volatile. Most flavonoids occur in the natural forms as glycosides, which cannot be analysed easily using EI and CI. This problem has been solved by the introduction of soft ionisation techniques described above, such as ESI (Yamashita 1984) and MALDI in recent years. Furthermore, ESI allow the hyphenation of mass spectrometric detectors with chromatographic separation devices such as HPLC. Using tandem MS experiments structural information can be obtained, which is important in the characterisation of metabolites formation.

A. Sample introduction

The two main methods for sample introduction are direct infusion using a syringe and flow injection, either with or without chromatographic separation.

Using direct infusion, the sample solution is infused directly into the ion source of the mass spectrometer, usually by the use of a syringe and a syringe pump. This method allows a long and thorough investigation of the sample. The major advantage is the possibility of several consecutive fragmentation steps, and is therefore useful for structural characterisation. In this thesis direct infusion of samples was employed with the analysis of the products derived from the direct interaction of catechin monomer and epicatechin dimer with nitrite in acid. The resulted in several fragmentation steps which enabled the identification of the

products. However, this method requires a purified sample, otherwise matrix effects and contaminants can lead to ion suppression effects and thereby decrease the sensitivity of the instrument. In addition, as the sample is normally infused with a flow rate of about 3 to 10 µl/min, a large sample amount is necessary for longer investigations (Kuhnle 2003).

Using flow injection reduces the sample amount required and allows the separation of the sample components before the mass spectrometric analysis. However, flow injection is less suitable for thorough analysis using tandem MS experiments, as it allows only a short investigation time for each signal (Kuhnle 2003).

B. Ion polarity

The hydroxyl groups of flavonoids are important in the ionisation of the compounds during mass spectrometry, as these groups are the main sites of protonation or deprotonation. A neutral or acidic environment is normally necessary to prevent decomposition of these compounds, which are unstable in a basic environment. Flavonoids can be detected in both positive and negative ion modes. While the positive ion mode often generates higher yields, the quality of the signal is better in the negative ion mode due to lower noise level (Kuhnle 2003). In addition, positive ion mode is more suitable for structure elucidation, whereas negative ion mode is better for the detection of compounds. Nevertheless, investigations regarding the polarity used are important to determine the optimal ionisation polarity for the compound of interest.

C. Ionisation method

The most commonly applied methods for LC-MS of flavonoids are electrospray and atmospheric pressure chemical ionisation (APCI) (Kuhnle 2003). In ESI the ions are preformed in solution and subsequently extracted in the spray. In contrast, during APCI the sample is evaporated by using high temperatures of up to 600°C and ionised by using a corona discharge. The main advantages of APCI over ESI are the increased range for flow rates and the potential to obtain ions from aqueous solutions even at flow rates well above 1ml/min. however, there are no studies comparing the best conditions for the investigations of flavonoids using APCI or ESI.

D. Fragmentation

Structural information of flavonoids can be obtained by fragmentation. The technique of choice with soft ionisation techniques is low-energy collision-induced

dissociation (CID). The fragmentation pattern of flavonoids has been proposed by Ma and colleagues (Ma 1997), and is the nomenclature generally used (Fig 2.9).

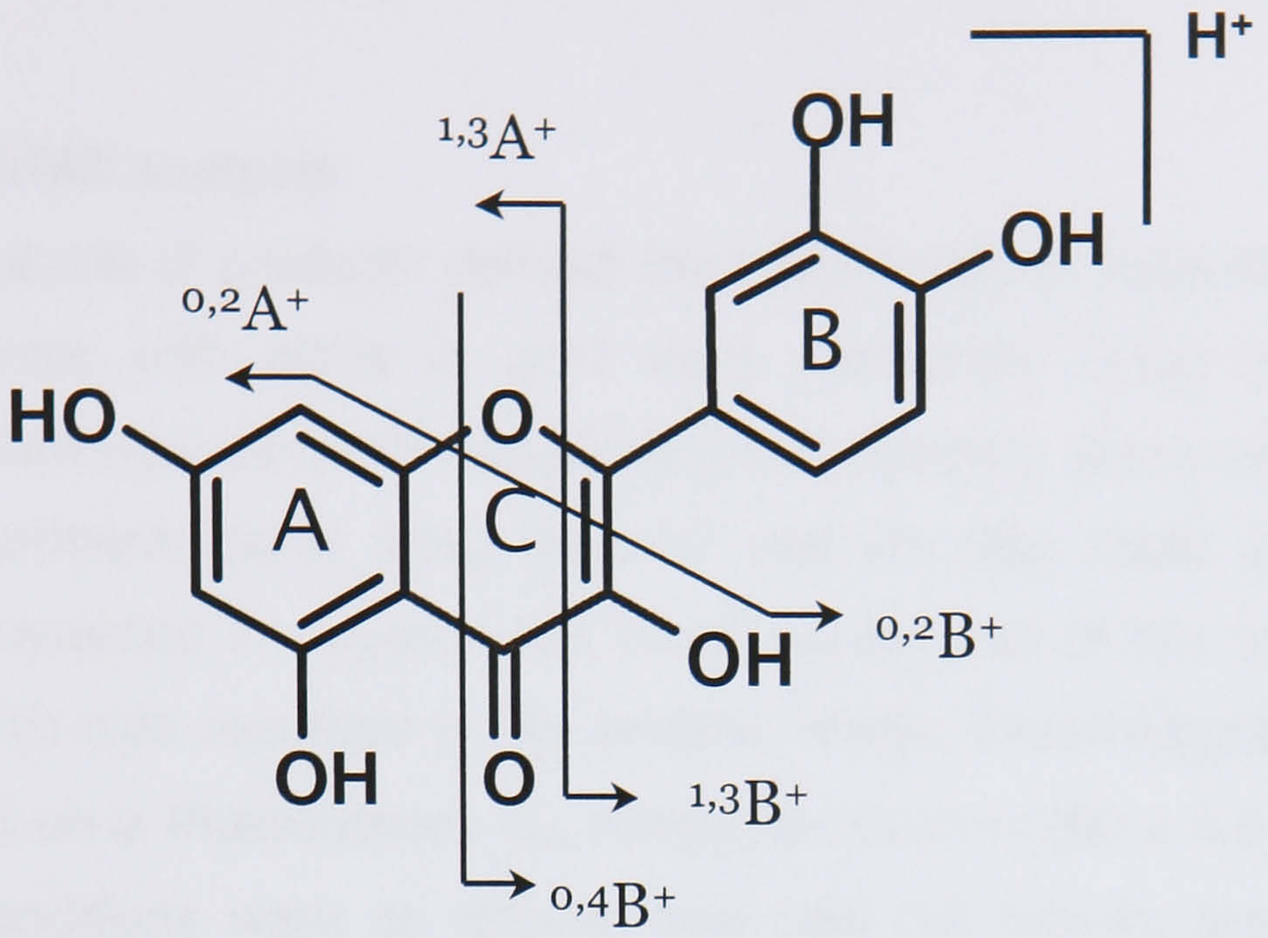


Fig 2.9 Nomenclature and selected product ions – derived from *retro-Diels-Alder* reaction – of protonated compounds under low energy CID as proposed in (Ma 1997). In $^{i,j}\mathbf{X}^{+}$, the superscript numbers (^{i,j}) denote the C-ring bond cleaved, the letter **X** describes the ring remaining in the fragment (taken from (Kuhnle 2003).

One of the most useful applications of tandem mass spectrometry in flavonoid research is the identification of the *in vivo* metabolites produced following pharmacokinetic studies. For instance, most *O*-methylated conjugates of flavonoids often show the neutral loss of the methyl group upon fragmentation. In addition, most *O*-methylated compounds generate several product ions, which allow identification and structure elucidation when a product ions scan is performed. As for the glucuronide conjugates, the 5- and 7-*O*-glucuronidated epicatechin exhibits a fragmentation pattern very similar to that of the aglycone, showing similar fragments including he metabolic modification (e.g. $[^{1,3}A^{+}$+ glucuronic acid$]^{+}$). However, this is not the case for all flavonoids, as the product ion spectrum of the quercetin-*O*-glucuronide exhibits the neutral loss of only glucuronic acid, but no other fragments, even though they are present in the product ion spectrum of the aglycone.

Conclusions

Mass spectrometry has become an important tool for the investigation of flavonoid *in vivo* metabolites due to its versatility and powerful identification of unknown compounds. It has provided valuable information on the detection of metabolites by

Materials and methods

using LC/MS, and the structural characterisation with tandem mass spectrometry, and therefore greatly advanced the field of flavonoid research.

2.2.1.7 LC-MS/MS analysis

LC-MS/MS analysis of products derived from interaction of catechin monomer and epicatechin dimer with nitrite in acid were performed using an Agilent 1100 HPLC/MS system equipped with refrigerated autosampler, binary HPLC pump, heat column compartment, diode array detector and ion trap mass spectrometer. All spectra were recorded in negative ion mode except that of the epicatechin dimer product II, which was recorded in the positive mode. Chromatographic separation was performed on a Phenomenex C_{18} Kingsorb column (250 x 4.6 mm i.d., 5 μM). The elution conditions were as follows: flow rate 0.8 ml/min, temperature of the column 40 °C. The mobile system used was a gradient of mobile phase A acetic acid/water (0.5/99.5 v/v) and mobile phase B acetic acid/acetonitrile (0.5/99.5 v/v), (min/% B): 0/5, 5/5, 40/50, 45/90. The eluent was monitored by photodiode array detection at 280 nm with spectra of products obtained over the 220-600 nm range and by mass spectrometry. The buffering reagent (methanol at a flow rate of 0.2 ml/min) was added *via* a tee in the eluent stream of the HPLC just prior to the mass spectrometer. Other conditions for analysis include: a capillary voltage of 3 kV; fragmentor exit offset of -60 V; a nebulising pressure of 9 psig, and the drying gas temperature at 350 °C. Data were collected on an HP ChemStation using both scan mode and selected ion monitoring. Spectra were scanned over a mass range of *m/z* 100-1000 at 1.96 s per cycle.

LC-MS/MS analysis of the remaining flavonoids was performed using a ThermoFinnigan (San Jose, CA, U.S.A.) LCQ Deca XP instrument with quadruple iontrap mass spectrometer. All spectra were recorded in positive ion mode. Chromatographic separation was performed on a Phenomenex C_{18} Luna column (50 x 2.0 mm) with the following elution conditions: flow rate 0.1 ml/min, temperature of the column 40 °C. The mobile system used was a gradient of mobile phase A: formic acid/water (0.1/99.9 v/v), and mobile phase B: 100% acetonitrile, (min/% B): 10/0, 50/25, 59/50, 60/0, 70/0.

2.2.2 Synthesis and purification of nitrosated catechins

Fig 2.10 outlines the methods used in the synthesis and isolation of nitrosated catechins (NCs).

Step 1 Synthesis of nitrosated catechin

↓

Step 2 Determination of suitable mobile phase system for detection of reference compound (NCs) using TLC (silica)

↓

Step 3 Flash chromatography

↓

Elution of compounds

↓

Step 4 Analysis of collected fractions for presence of NCs by TLC (silica)

↓

Step 5 Isolation of NC from fraction(s) by preparative TLC

↓

Step 6 Determination of purity of the isolated NC's by TLC

↓

Step 7 Further purification of NC's by preparative HPLC

Fig 2.10 The synthesis and isolation of nitrosated catechins.

Materials and methods

2.2.2.1 Interaction of catechin with acidic nitrite to synthesise nitrosated catechins

Stock solutions of $NaNO_2$ (50 mM) were prepared in water and catechin monomer (50 mM) was prepared in methanol. Equimolar concentration (20 mM) of catechin monomer and $NaNO_2$ were incubated in 0.5 M HCl at 37°C for 2h, and the reaction was terminated by placing the reaction mixture on ice. The methanol content was removed by rotary evaporation of the samples at 30°C. Samples were then freeze-dried for 72h. The weight of the resulting powder, consisting of the nitrosated catechin preparation (NC prep), was approximately 1.5g.

2.2.2.2 Detection of nitrosated catechins by TLC

Commercially available TLC aluminium sheets were used for all analytical experiments. The sorbent in normal phase sheets was silica gel 60 with inherent fluorescence at 254 nm, whereas C_{18} octadecyl was used in reverse phase analysis (Merck Ltd., Lutterworth, Leicester). All analytical TLC separations were performed by standard linear development in a customary trough chamber.

In order to develop a suitable TLC system for the detection of nitrosated catechins, purified standards of nitrosated catechin 1 (NC1) and nitrosated catechin 2 (NC2) were required. A small amount of NC1 and NC2 standards were prepared using preparative HPLC as described in section 2.2.1.5. Initial studies to achieve detection of NC standards on TLC involved testing several mobile systems. Nitrosated catechin standards were applied to a series of small plates, and each plate was developed in different test mobile systems. Separation of NC standards was then determined for each system using various detection methods.

The mobile systems tested were:

1. Methanol : Dichloromethane : Water (10 : 10 : 1)
2. Methanol : Dichloromethane (80 : 20)
3. Ethyl acetate : Formic acid : Acetic acid : Water (100 : 11 : 11 : 27)
4. Methanol : Hydrochloric acid (5N) : Water (50 : 50 : 0.1)

Mobile systems 5 to 8 were prepared by mixing different proportions of:

A: Methanol : Hydrochloric acid (5N) : Water (5 : 0.1 : 95)

B: Acetonitrile : Hydrochloric acid (5N) : Water (50 : 0.1 : 50)

Materials and methods

5. A (100)
6. A : B (75 : 25)
7. A : B (50 : 50)
8. A : B (25 : 75)

Normal phase silica gel plates were used with mobile phase systems 1 to 3, and reverse phase C_{18} plates were used with mobile phase systems 4 to 8. The most common visualisation method is the use of fluorescent layers that enable examination under UV light for the detection of all quenching compounds. The normal phase silica plates used in this study were manufactured with inherent fluorescence at 254 nm. Compounds containing conjugated double bonds can be detected by visualisation under UV light at 254 nm. Table 2.1 lists the detection methods using chromogenic sprays for the detection of NCs. Chromatograms were always sprayed in a fume hood.

Spraying reagent	*Treatment*	*Types of compounds detected*
• Dragendorff's reagent	Examine in daylight	Nitrogen containing compounds give orange zones on yellow background
• Anisaldehyde spray	Heat at 105°C for 10 min. Observe in daylight	Flavonoids give brown zones
• Naturstoff reagent	Examine under UV light 365 nm	Flavonoids give fluorescent zones
• 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical[1]	Examine in daylight	Antioxidants appear as yellow spots against a purple background.

Table 2.1 Chromogenic spray detection systems for layer chromatograms. [1] $DPPH^{\bullet}$ is a radical with a purple colour, which turns colourless when quenched. See Appendix 1 for details of the compositions of the chromogenic sprays. Adapted from (Raman 1998).

Materials and methods

2.2.2.3 Flash chromatography

Once a suitable mobile system was determined, crude fractionation of the NC prep was achieved using flash chromatography.

A. Sample preparation

Approximately 0.5g of NC prep was used for the isolation of NCs using a 20g C_{18} flash column. The sample was mixed with silica gel prior to sample application to enable better separation. The ratio of sample to silica gel was usually 1:1 or 1:3. Although this was a reverse phase system, sample preparation can be carried out with normal phase silica gel.

Nitrosated catechin prep was dissolved in 1.5 ml methanol and was transferred to an evaporating dish on a hot plate. Silica gel (1.5g) was added and the mixture stirred until it dried completely. The dried powder was then applied to the column using a filter funnel to achieve an even surface. A frit was fitted onto the column before the plunger was inserted.

B. Flash chromatography

Since the polarities of methanol and acetonitrile (mobile phases for HPLC) are quite similar, using a combination of these two solvents on flash chromatography system may not provide a good separation of nitrosated catechins. The mobile systems were therefore changed to: A – 0.1% hydrochloric acid (5N) in water; and B – pure methanol. A step gradient system was used and the fractions collected are shown in Table 2.2.

Fractions	*% A*	*% B*	*Flow rate (ml/min)*	*Time (min)*
1 → 8	95	5	70	10
9 → 12	95	5	80	5
13 → 21	85	15	75	10
22 → 25	70	30	75	5
26, 27	45	55	75	5

Table 2.2 Conditions for the fractionation of NC prep using flash chromatography.

The remaining sample of NC prep was fractionated using a modified version of the above method. A more linear gradient was used and the flow rate was reduced to 70 in order to lower the pressure of the column. NC prep (0.5g) was used and was

prepared according to the method described above. Table 2.3 depicts the conditions of the flash chromatography and the fractions collected.

Fractions	*% A*	*% B*	*Flow rate (ml/min)*	*Time (min)*
1 → 6	95	5	70	10
7 → 12	85	15	70	10
13 → 18	75	25	70	10
19 → 23	50	50	70	10
24 → 26	0	100	70	5
27	0	100	70	2

Table 2.3. Modified conditions for the fractionation of NC prep using flash chromatography.

C. Analysis of fractions for nitrosated catechin

Fractions obtained using flash chromatographic separation were analysed for the presence of NCs using the TLC system described in Section 2.2.2.2. This involved using normal phase silica gel 60, F_{254} with the mobile system: Ethyl acetate : Formic acid : Acetic acid : Water (100 : 11 : 11 : 27) (Males and Medic-Saric 2001).

The presence of NCs was determined by examining the plates under short wavelength (254 nm) UV light. Nitrosated catechin contains conjugated double bonds and therefore would quench the fluorescence at 254 nm. Once the fractions containing nitrosated catechins were identified, they were freeze-dried and analysed by TLC to confirm the presence of NCs before further isolation using preparative TLC.

2.2.2.4 Preparative TLC

A. Determination of mobile system

The TLC system optimised for the analytical analysis of NCs resulted in good separation of NCs from catechin (Section 2.2.2.2). Since the amount of sample loaded onto prep TLC plate is often greater than that for analytical TLC, resolution of compound is usually compromised. Consequently, addition mobile systems were tested for better separation of nitrosated catechins from catechin.

Three mobile systems were tested:

1. Ethyl acetate : Methanol : Water : Toluene (200 : 27 : 10 : 5)
2. Ethyl acetate : Methanol : Water : Toluene (113.5 : 113.5 : 10 : 5)

Materials and methods

3. Ethyl acetate : Methanol : Water : Toluene (27 : 200 : 10 : 5)

B. Preparation of prep TLC plates

Although commercially available TLC plates are better quality and more robust than 'home-made' plates, all preparative TLC were carried out using plates prepared in the laboratory due to the large number of plates required and thus the cost involved.

Silica gel PF_{254} (Merck) was used as the stationary phase in all experiments. The plates were prepared by mixing 80g silica gel in 200 ml H_2O. The suspension was spread evenly (using a plate spreader) by pouring into a trough with a gap of 1mm at the base of the trailing edge onto five glass plates. Once the suspension was dried, the plates were heated in an oven at 105 °C for 30 min to activate the sorbent.

C. Preparative TLC

The sample collected using flash chromatography was applied horizontally across the plate approximately 2 cm from the plate edge. To avoid oxidation of NCs, the TLC tank was wrapped in aluminium foil and saturated with nitrogen gas during plate development using ethyl acetate: formic acid: acetic acid: water (110: 11: 11: 27) as the mobile system. Non-destructive detection of NCs was achieved by examination under UV light at 254 nm. The band of interest was immediately removed from the plate into glass test tube. Nitrosated catechins were extracted from the silica sorbent with methanol. The extraction procedure was repeated three times to ensure maximum extraction of nitrosated catechins. Methanol was subsequently removed from the NCs extract by rotary evaporation.

Following prep TLC the resulting sample was analysed using analytical TLC for the presence of NCs, and to determine the purity of the sample (as described above).

D. HPLC analysis

Reverse-phase HPLC was employed for more accurate determination of the purity of the NCs extract. Prior to HPLC analysis samples were subjected to a cleaning procedure for the removal of silica:

- Centrifugation at 13,000 rpm for 30 min
- Filtration with cotton wool

Analysis was carried out using a Hewlett Parkcard 1100 HPLC system with an auto-injector, auto-sampler, and diode array detector linked to a HP ChemStation for data collection and processing. The sample was injected onto a Nova-Pak C_{18} (250 mm x 4.6 mm i.d., 4 μm) column using an autoinjector. The mobile system consisted of A – 10% methanol, 0.1% 5N HCl in water, and B – 50% acetonitrile, 0.1% 5N HCl in water. The following gradient system was used (min/% acetonitrile): 0/5, 30/50, 35/50, 36/5, 40/5 with a flow rate of 0.7 ml/min.

2.2.2.5 Preparative HPLC

Fractions collected from flash chromatography were further purified using preparative HPLC. A reverse phase Luna C_{18} column (250 mm x 10 mm i.d., 5 μm) with mobile system consisting of A – 10% methanol, 0.1% 5N HCl in water, and B – 50% acetonitrile, 0.1% 5N HCl in water was used. The gradient system used was (min/% acetonitrile): 0/5, 30/50, 35/50, 36/5, 40/5 with a flow rate of 2 ml/min. Nitrosated catechins were collected by monitoring the eluent at 280 nm. The samples collected were freeze-dried following semi-preparative HPLC, and an aqueous 10 mM stock solution prepared; which was then aliquoted into 0.5 ml samples and stored at -80°C.

2.2.3 Absorption and metabolism of procyanidins – Caco-2 cell model

2.2.3.1 Culture of Caco-2 cells

Caco-2 cells obtained from the European Collection of Animal Cell Culture (Salisbury, UK) were cultured in 75-cm^2 Nunc flasks and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with Glutamax-I, supplemented with 10% heat-inactivated bovine serum, 1% non-essential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 5% (v/v) CO_2 and subcultured after trypsinisation (0.5% trypsin/2.6 mM EDTA). Cells used in all experiments were at passage 25 to 50. Culture media were replaced every 2-3 days and cells passaged at least once a week.

2.2.3.2 Assessment of permeability and metabolism

Caco-2 cells were seeded on Transwell inserts (polycarbonate membrane, 12 mm i.d., 0.4 μm pore size, Corning Costar Corp., UK) at a density of $1x10^5$ cells/cm^2 and cultured for 21 days. Caco-2 cells are derived from a human colonic adenocarcinoma (Fogh 1977), and undergo differentiation in culture to form

monolayers that resemble the small intestine when grown for 21 days (see Section 1.1.4.1 for detail). Cells are proliferative until 10 days in culture (reaching confluence) and are fully differentiated after 14 days. The formation of monolayers with tight junctions was monitored by measuring the transepithelial electrical resistance (TEER) values across the monolayers using a Millicell-ERS Voltohmmeter (Millipore Corp, Bedford, MA). Monolayers with TEER values > 350 Ω cm^2 in culture medium were selected for transport experiments. For both apical to basolateral (A to B) and basolateral to apical (B to A) transepithelial permeability of flavonoids was measured at 25 μM for 2h. All compounds were diluted from 10mM stock solutions in methanol except quercetin (in DMSO).

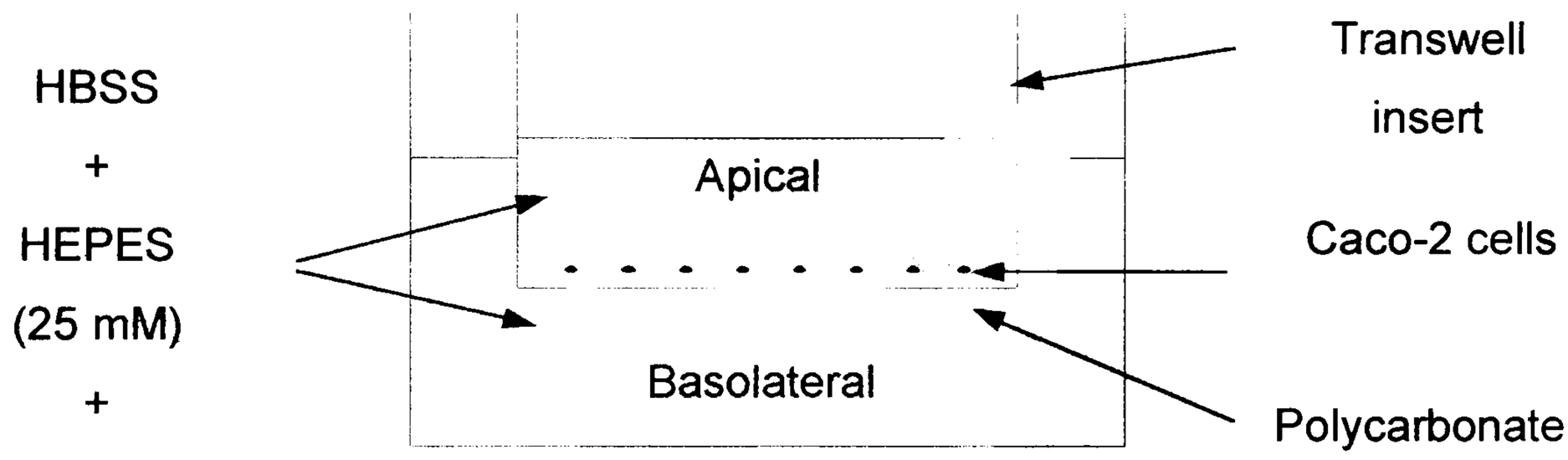


Fig 2.11 Diagrammatic representation of the Caco-2 permeability model. Caco-2 cells are grown as a monolayer in transwell inserts, with the apical side of the membrane representing the lumen of the small intestine.

A. Apical (A) to basolateral (B) permeability

The A to B permeability of 3-nitrotyrosine, nitrosated catechins, catechin, chrysin, cyanidin-3-rutinoside, epicatechin, epigallocatechin gallate, epicatechin gallate, hesperetin, hesperetin-7-rutinoside, kaempferol, naringenin, naringenin-7-glucoside, pelargonidin-3-glucoside, procyanidins dimer B2, quercetin, resveratrol and rosmarinic acid were studied. Growth medium in the apical and basolateral sides of the inserts were replaced with 0.5 and 1 ml transport buffer (Hanks Balanced Salt Solution (HBSS) containing 25 mM HEPES and 0.1% BSA) and incubated for 30 min. After 30 min preincubation, the transport buffer in the apical side was replaced with fresh transport buffer containing flavonoids (25 μM). The paracellular transport marker [^{14}C]-mannitol (0.01μCi/ml) was added to the apical side for the assessment

Materials and methods

of monolayers integrity during the transport study. Aliquots of the basolateral medium were assayed for [^{14}C]-mannitol flux at the end of the experiment. All experiments were performed at 37°C on a Wesbart shaking plate set at 200 rpm (Wesbart Ltd, UK) to minimise unstirred water layer effects (Avdeef 2001). Samples from both sides of the cell monolayers were collected at the end of experiments and analysed by HPLC for the presence of flavonoids. The radioactivity of samples was also determined for evidence of [^{14}C]-mannitol transport by scintillation counting. Aliquots were removed and added to scintillation cocktail and the amount of [^{14}C]-mannitol determined using a Beckman scintillation counter (Global Medical Instrumentation, Inc., USA).

B. Basolateral to apical permeability

The B to A permeability of catechin, epicatechin, hesperetin and quercetin were investigated. This was carried out as described above with the exception that flavonoids and [^{14}C]-mannitol were added to the basolateral side. Samples from both sides of the cell monolayers were collected at the end of experiments and analysed by HPLC for the presence of flavonoids and scintillation counting for radioactivity of [^{14}C]-mannitol, as described above.

2.2.3.3 HPLC analysis

Reverse phase HPLC analysis was performed on a Millennium HPLC system (Waters Corp.) with a Nova-Pak C_{18} column (250 mm x 4.6 mm i.d., 4 μm) and guard column (15 x 4.6 mm i.d., 4 μm). Mobile phase A consisted of methanol/water/5N HCl (5/94.9/0.1 v/v/v) and mobile phase B of acetonitrile/water/5N HCl (50/49.9/0.1 v/v/v). The following gradient system was used (min/% acetonitrile): 0/0, 5/0, 40/50, 60/100, 65/100, 65.1/0 with a flow rate of 0.7 ml/min. The eluent was monitored by photodiode array detection at 280 nm with spectra of products obtained over the 220-600 nm range.

For the determination of glucuronide conjugates, samples (180 μl) were treated with β-glucuronidase (20 μl, 1000 units/ml) for 2h at 37°C in a 0.1 M sodium acetate buffer, pH 3.8. Released compounds were analysed by HPLC according to the procedure described above. The amount of glucuronide present in the samples was

calculated based on the difference in flavonoid aglycone content before and after enzyme treatment.

2.2.3.4 Permeability calculations

Following HPLC analysis of flavonoid content in the apical and basolateral compartments, the apparent permeability (P_{app}) (i.e. with no correction for the influence of the filter (P_{filter}) supporting the cell monolayer) (Youdim 2003) was calculated in two ways:

It has been customary to use the following equation for permeability calculation of flavonoids in the literature (Galijatovic *et al.,* 1999, Vaidyanathan and Walle 2001, Walle 2003).

$$P_{app} = V_R / (A.C_{D0}) \times (\Delta C_R / \Delta t) \quad (1)$$

Where V_R is the volume of buffer in the receiving compartment (cm^3), A is the surface area of the membrane (cm^2), C_D is the donor concentration at start of experiment, and $\Delta C_R / \Delta t$ is the concentration of compound transferred to receiver compartment over time (sec).

A mathematically equivalent version of the above equation (1) can be generated using mass terms instead of concentration. This has the advantage that mass balance can be readily followed (Youdim 2003).

$$P_{app} = V_D / (A.M_D) \times (\Delta M_R / \Delta t) \quad (2)$$

Where V_D is the volume of buffer in the donor compartment (cm^3), A is the surface area of the membrane (cm^2), $\Delta M_R / \Delta t$ is the amount of compound transferred to the receiver compartment over time (sec).

The above equations assume that there is no mass loss to the cell membrane. However, with some lipophilic compounds membrane retention can be quite substantial. The following equation (3) corrects for compound retention in the cell monolayer.

$$P_{app} = V_D / (A.M_D - M_{cells}) \times (\Delta M_R / \Delta t) \quad (3)$$

where M_{cells} is the amount of compound retained by the membrane.

In the present study, P_{app} of flavonoids was calculated using equations 2 and 3. An example of the calculation can be found in Appendix 3.

2.2.3.5 Partition coefficient determination

The lipophilicity of flavonoids was determined by calculating their calculated octanol-water partition coefficient (cLog P) using the online program LogKow (KowWin) (available at http://esc.syrres.com/interkow/kowdemo.htm). This program uses fragmental analysis of the compound structure for its prediction and the values obtained show a high correlation with quoted experimental values (p = 0.98) (Meylan and Howard 1995).

2.2.3.6 P-glycoprotein assay

Cell culture

Madin-Darby canine kidney type II cells transfected with the full-length cDNA for human multidrug resistance gene 1 (MDCK-MDR1) were kindly provided by R. Melarange (GlaxoSmithKline, Ware, UK). The cells were cultured in DMEM containing glucose (4.5g/L), supplemented with 10% foetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, and maintained at 37°C in a humidified atmosphere of 5% (v/v) CO_2. Cells used in all experiments were at passage 90 to 100. Culture media were replaced every 2-3 days and cells passaged at least once a week.

Interaction with P-glycoprotein

The effect of various inhibitors on P-glycoprotein (P-gp) activity was first evaluated to determine a suitable positive control. Inhibition of P-gp activity was investigated using rhodamine 123 (R123) as a substrate of P-gp (Batrakova *et al.,* 2003, Fontaine *et al.,* 1996). The modulatory effects of compounds towards P-gp function were determined by their influence on cellular accumulation of R123 in MDCK-MDR1 cells.

MDCK-MDR1 cells were seeded onto 96 well plates (Corning Costar Corp., UK) at a density of $5x10^4$ cells/well for 24 hours. The effect of PSC833, GF120918,

cyclosporin A and verapamil on P-gp activity were studied by co-incubating 50 μM of inhibitors with 10 or 20 μM R123 in transport buffer (HBSS containing 25 mM HEPES and 0.1% BSA) for 60 min at 37°C in a humidified atmosphere. After incubation, cells were rapidly washed three times with ice-cold phosphate-buffered saline (PBS) to remove excess R123. The cells were then lysed by incubation with 100 μl 2% triton-X100 solution for 60 min at room temperature. The amount of R123 taken up by MDCK-MDR1 cells was analysed using a fluorescence plate reader (Molecular Devices, California, USA) with excitation wavelength at 500 nm and emission wavelength at 550 nm.

The modulatory effects of catechin, epicatechin, hesperetin and quercetin (12.5-100 μM) co-incubation with R123 on P-gp function, were determined as described above. Cyclosporin A was found to show the greatest inhibition of P-gp, and was therefore used as a positive control in all experiments.

2.2.4 Isolated rat small intestine model

Male Sprague-Dawley rats weighing 220-250 g were bred at the Comparative Biology Unit at the Royal Free and University College Medical School. All procedures were carried out within the Home Office guidelines of the Animal (Scientific Procedures) Act, 1980. The rats were housed in temperature-controlled rooms with 12h light/dark cycle, and provided with standard rat chow (Diet RM1, SDS Ltd, Witham, Essex, UK) and water, *ad libetum*.

2.2.4.1 Assessment of permeability and metabolism

Absorption studies were conducted using the *in vitro* intestinal preparation of Fisher and Gardner (Fisher and Gardner 1974). Each rat was deeply anaesthetised with pentobarbital sodium (90 mg/kg i.p.) during the removal of the small intestine via an abdominal incision. Sections of jejunum were cannulated and the lumen rinsed with Krebs bicarbonate buffer. The bicarbonate buffer consisted of Krebs bicarbonate saline solution (HCO_3^-: 25 mM; Na^+: 143 mM; Cl^-: 133.7 mM; K^+: 5.9 mM; HPO_4^-: 1.2 mM) containing Ca^{2+} (2 mM) and Mg^{2+} (1.2 mM). The rats were kept alive during removal of the jejunum and euthanised immediately after removal of the tissue. The jejunum was then suspended in a chamber containing liquid paraffin and maintained at 37°C. The lumen was perfused for 40 min with a segmental flow (perfusion of buffer interspersed with bubbles of the gas mixture) of bicarbonate buffer at pH 7.4,

containing 28 mM glucose, equilibrated with 95% O_2, 5% CO_2 to allow fluid absorption to reach a steady state. A segmental flow was used to deliver oxygen to sustain the viability of the rat jejunum as well as reduce the thickness of the unstirred water layer (Fisher and Gardner 1974, Helliwell *et al.,* 2000). Flavonoids were then added to the buffer and perfused through the system in a single pass fashion at a flow rate of 2ml/min, up to 80 min (viability of tissues decrease after 90 min) (Kuhnle *et al.,* 2000). During perfusion, absorbed fluid dropped through the liquid paraffin to the base of the chamber and was collected at 20 min intervals for analysis. Samples were immediately acidified by addition of 20 µl 5N HCl to prevent degradation (oxidation) of flavonoid during storage.

The advantage of this model in the assessment of flavonoid uptake by the small intestine is that serial measurements can be made using defined intestinal segments over short intervals. Furthermore, the solutes under investigation appear on the serosal surface in the same form as if they were transferred to the mesenteric circulation. This model also excludes the action of intestinal microflora on flavonoid metabolism.

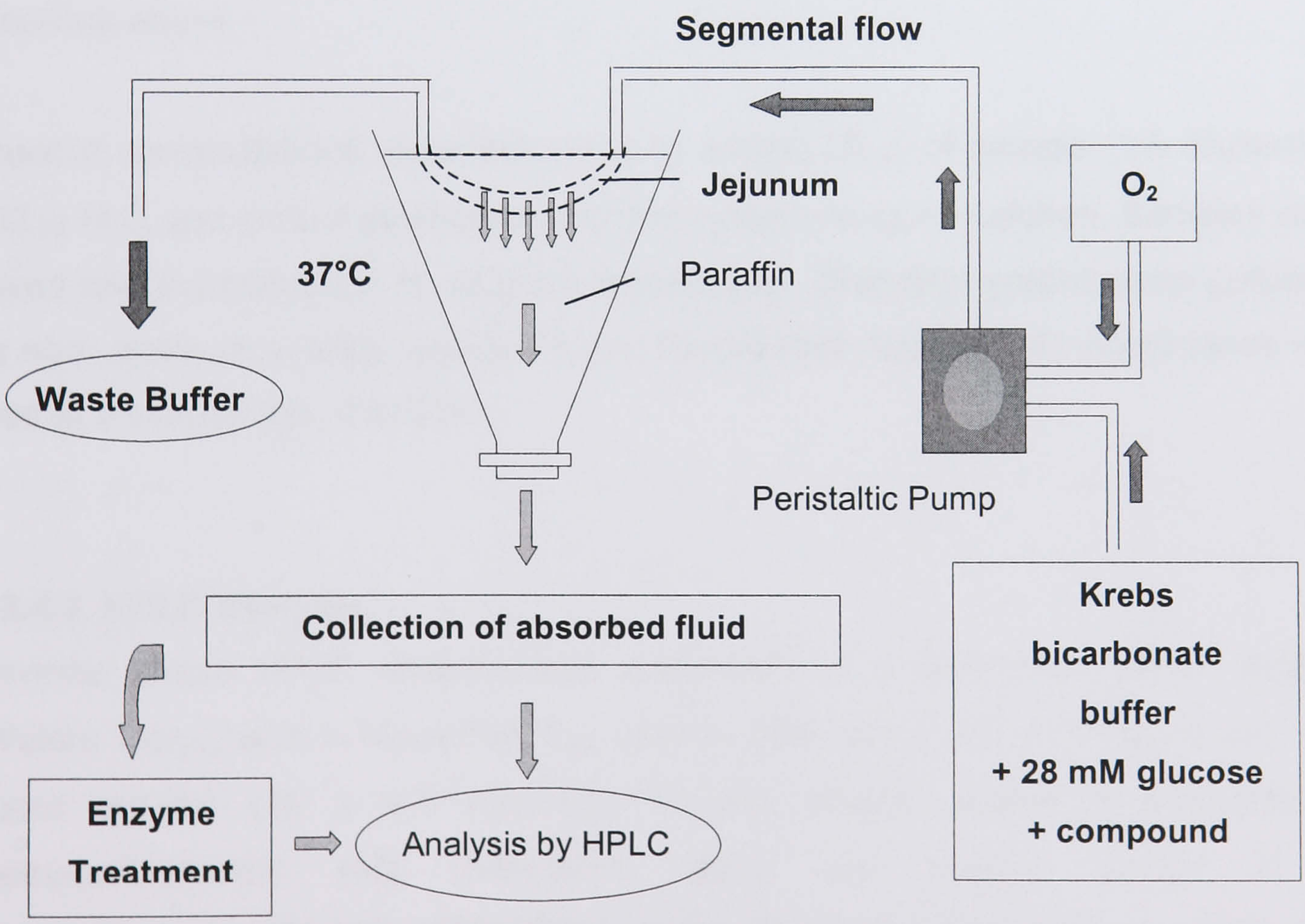


Fig 2.12 Diagrammatic representation of the isolated rat small intestine perfusion model. Buffers and compounds are perfused for up to 80 min at a rate of 2 ml/min at 37°C using a peristaltic pump. Serosal fluid was collected from the bottom of the paraffin chamber and analysed by HPLC. (Adapted from (Kuhnle *et al.*, 2000).

Materials and methods

2.2.4.2 Assessment of tissue viability

The viability of the isolated jejunum during the course of the experiment was determined by comparing the concentration of glucose in the perfusion buffer and the serosal fluid at 40 and 80 min after the initial 40 min equilibration period. The concentration of glucose present was determined using the glucose oxidase colourimetric assay kit (Sigma-Aldrich Chemical Company, Dorset, UK), which involves the enzymatic conversion of glucose to gluconic acid and hydrogen peroxide:

$$\text{Glucose} + H_2O + O_2 \xrightarrow[\text{Glucose Oxidase}]{} \text{gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{o-dianisidine} \xrightarrow[\text{Peroxidase}]{} \text{oxidised o-dianisidine (brown)}$$

The indicator used was oxidised o-dianisidine formed from o-dianisidine and hydrogen peroxide under the catalytic effect of peroxidase according to the reactions above.

Glucose concentrations were measured by adding 25 µl of sample (1:5 dilution) to 500 µl H_2O and 4 ml of peroxidase/glucose oxidase enzyme solution. Samples were mixed and incubated for 1h at room temperature. Standard graphs were prepared for each assay in a range between 5 and 15 mM (see Appendix 4). Absorbance was read at a wavelength of 450 nm.

2.2.4.3 HPLC analysis

Reverse phase HPLC analysis was performed on a Millennium HPLC system (Waters Corp.) with a Nova-Pak C_{18} column (250 mm x 4.6 mm i.d., 4 µm) and guard column (15 x 4.6 mm i.d., 4 µm). Mobile phase A consisted of methanol/water/5N HCl (5/94.9/0.1 v/v/v) and mobile phase B of acetonitrile/water/5N HCl (50/49.9/0.1 v/v/v). The following gradient system was used (min/% acetonitrile): 0/0, 5/0, 40/50, 60/100, 65/100, 65.1/0 with a flow rate of 0.7 ml/min. The eluent was monitored by photodiode array detection at 280 nm with spectra of products obtained over the 220-600 nm range.

For the determination of glucuronide conjugates, samples (180 μl) were treated with β-glucuronidase (20 μl, 1000 units/ml) for 2h at 37°C in a 0.1 M sodium acetate buffer, pH 3.8. Released compounds were analysed by HPLC according to the procedure described above. The amount of glucuronide present in the samples was calculated based on the difference in flavonoid aglycone content before and after enzyme treatment.

A number of minor adjustments to the gradient were made when analysing catechin samples, as this flavonoid was found to co-elute with the amino acid tryptophan. The gradient systems tested (min/% acetonitrile) were:

i) 0/0, 5/0, 50/50, 65/100, 65.1/0

ii) 0/0, 5/0, 55/50, 65/100, 65.1/0

iii) 0/0, 5/0, 20/25, 50/50, 65/100, 65.1/0

The effect of solid phase extraction on the removal of tryptophan was also examined. A mixture containing 50 μM catechin and 50 μM tryptophan in Kreb's buffer was prepared, and the removal of tryptophan was tested using a C_{18} Sep-pak solid-phase extraction (SPE) column (Waters Corporation, Milford, MA, USA). The column was conditioned using 2 ml of methanol followed by 2 ml of water. A 500 μl aliquot of the mixture was then passed through the column, and then washed with 2x1 ml of water. An aliquot of 100 μl of the collected sample was injected onto the HPLC column for analysis (see also Section 6.2.3).

Results from the small intestinal absorption experiments were expressed as the amount (ng) of test compound absorbed per cm of jejunum per min. See appendix 4 for an example of the calculation.

2.2.5 Biological properties of procyanidins and nitrosated products

2.2.5.1 Culture of human fibroblasts (FEK4 cells)

Normal human dermal fibroblasts derived from foreskin (FEK4) were used between passages 9 and 15. The cells were maintained in Earle's minimum essential medium, supplemented with 15% foetal calf serum (PAA Laboratories Ltd., Somerset, UK), 1% L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin

Materials and methods

and 2.7% sodium bicarbonate. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. Culture media were replaced every 2-3 days and cells passaged at least once a week.

2.2.5.2 Assessment of cytotoxicity

A. Effect of nitrosated catechins on Caco-2 cell proliferation

Cells were seeded at 4500 cells per well in 96 well plates, and were maintained as described in Section 2.2.3.1. Catechin and NCs (3-30 μM) were added 24h after seeding to the culture medium. Fresh media containing catechin and NCs were added every 48h. Control wells contained methanol at a final concentration identical to that in wells supplemented with 30 μM catechin and NCs. The final concentration of methanol in all samples did not exceed 0.1%. Cells were harvested after 7, 14 and 21 days in culture, and the cell biomass was determined using a commercially available kit (Sigma-Aldrich Chemical Company, Dorset, UK). Cells were fixed by addition of 25 μl ice-cold acetic acid at 4°C for 1h and the total biomass was determined by staining with sulforhodamine B (SRB). An increase or decrease in the number of cells (total biomass) results in a concomitant change in the amount of dye incorporated by the cells in the culture, which was measured at 490 nm, and subtracted from the background absorbance at 690 nm, using a SPECTRAmax® 190 micro-plate photometer (Molecular Devices, California, USA). This indicates the degree of cytotoxicity caused by the test compounds (Skehan *et al.,* 1990).

A standard curve of cell number vs. absorbance of sulforhodamine B was constructed from 0 – $4.8x10^5$ cells/well for data analysis (see Appendix 5). The relationship between number of cells (protein content per well) and absorbance is linear from 0 to 240,000 cells per well.

B. Toxicity of nitrosated catechins on FEK4 cells

For the toxicity studies, fibroblasts were seeded in 24-well plates at 1 x 10^4 cells/well and grown for 72h before use. Cells were pre-treated for 18h with 3-50 μM nitrosated catechins and catechin (30 μM) as a comparison, prepared in fresh media from 10 mM stock solutions. After pre-treatment, cells were incubated in fresh media for 24h before their viability was evaluated using the MTT colorimetric assay. Briefly, cells were washed twice with sterile PBS before addition of the MTT solution and incubated for 2h at 37°C. MTT (0.5 mg/ml) was dissolved in sterile HEPES-buffered

incubation medium (HBM), pH 7.4 (5 mM Hepes/154 mM, NaCl/4.6 mM, KCl/2.3 mM, $CaCl_2$/33 mM, glucose/5 mM, $NaHCO_3$/1.1 mM, $MgCl_2$/1.2 mM and Na_2HPO_4). The MTT solution was subsequently replaced with DMSO (0.5 ml) to facilitate dissolution of the formazan crystals formed during the MTT incubation period. The absorbance was then measured using a SPECTRAmax® 190 micro-plate photometer (Molecular Devices, California, USA) at 495 nm and compared with respective controls (no compound present). The absorbance was always measured within 30 min of adding the DMSO.

2.2.5.3 Protection against H_2O_2-induced cytotoxicity

Cells were cultured as described in Section 2.2.5.1. Following 18h pre-treatment with NCs and catechin (3-30 μM), cells were washed twice with PBS (to prevent direct extracellular interactions between compounds and H_2O_2) prior to addition of H_2O_2 (75 μM). Exposure to H_2O_2 took place in sterile HBM buffer for 120 min. H_2O_2 was replaced with fresh media for 24 h before the cell viability was evaluated using the MTT colorimetric assay, as described in the previous section.

2.2.6 Statistical analysis

Data were expressed as mean ± Standard error mean (SEM) values. Statistical comparisons were made using an unpaired, two-tailed Student's *t* test using GraphPad Instat version 3.05 for Windows 95 (GraphPad Software, San Diego California USA, www.graphpad.com). One-way ANOVA with Tukey-Kramer multiple comparison post test was performed when appropriate using GraphPad Instat version 3.05 for Windows.

CHAPTER THREE

Interaction of cocoa flavonoids with acidic nitrite-generated reactive nitrogen species

3.1 OBJECTIVES

Nitrate is present in drinking water and green leafy vegetables. As such our diet contains a relatively large amount of nitrate, with a daily consumption of approximately 52 mg (Ysart *et al.,* 1999). Consumption of nitrate can lead to the production of various reactive nitrogen species (Leaf *et al.,* 1989, McKnight *et al.,* 1997) via its conversion to nitrite by bacteria in our bodies Nitration of protein tyrosine residues is one of the mechanisms whereby RNS contributes to the pathogenesis of diseases. Enhanced levels of nitrotyrosine have been detected in many diseases, including Alzheimer's disease (Smith *et al.,* 1997), atherosclerosis (Beckman 1994), myocardial inflammation (Kooy *et al.,* 1997), lung cancer (Pignatelli *et al.,* 2001) and gastric cancer (Goto *et al.,* 1999). Recently, the ability of flavonoids to scavenge RNS has been demonstrated in various studies (Arteel and Sies 1999, Haenen and Bast 1999, Oldreive *et al.,* 1998, Pannala *et al.,* 1997, Pannala *et al.,* 1998), implicating their potential role to provide protection against diseases relating to RNS. Thus the purpose of this study was:

1) To investigate the ability of monomeric and dimeric cocoa procyanidins to scavenge RNS derived from acidic nitrite (akin to conditions in the stomach) by examining their ability to inhibit tyrosine nitration *in vitro*.

2) To establish the reaction mechanism underlying the protective ability of procyanidins by:

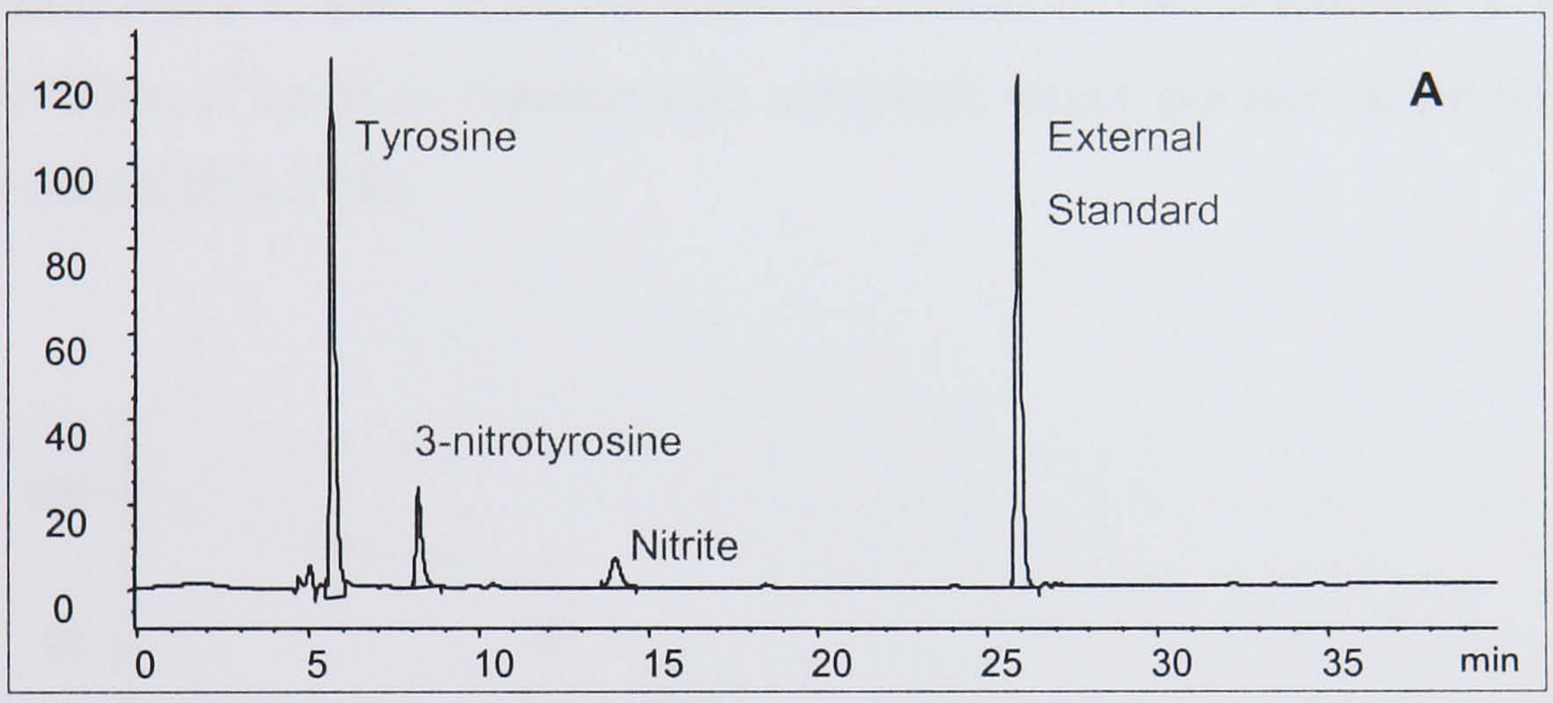
   - Examining the direct interactions between procyanidin and acidic-nitrite derived RNS and investigating the nature of the products using LC-MS/MS.

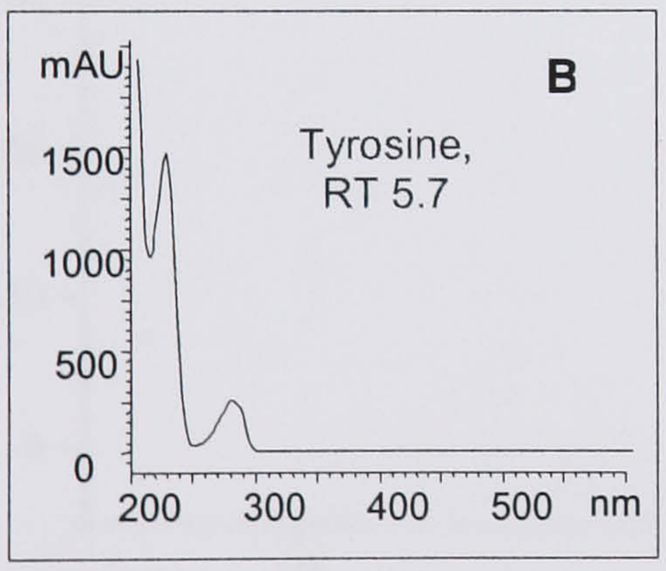
   - Comparing the products derived from related structures including methylation of the B ring to block the catechol structure and the influence of C ring alteration on the reaction profile.

3.2 RESULTS

3.2.1 Interaction of tyrosine with acidic nitrite

The formation of 3-nitrotyrosine from acidic nitrite-mediated tyrosine nitration was investigated by HPLC analysis and is shown in Figs 3.1. The first peak with retention time 5.7 min is the tyrosine peak and the peak with RT 8.2 min is the 3-nitrotyrosine peak. The last peak represented the external standard that was added prior to HPLC analysis. 3-Nitrotyrosine formed from 400 μM nitrite and 400 μM tyrosine comprised 11.5 ± 1.7 μM, consistent with literature reports (Oldreive *et al.*, 1998).





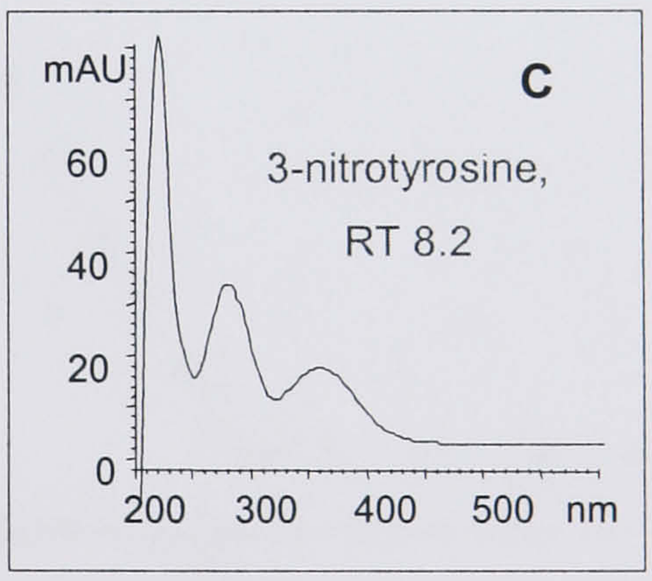


Fig 3.1 Reverse phase HPLC analysis of interaction between 400 μM tyrosine and 400 μM nitrite in 0.5M HCl. (A) Chromatogram of tyrosine nitration. At 400 μM concentration of tyrosine and $NaNO_2$, the average concentration of 3-nitrotyrosine formations was calculated to be approximately 11.5 μM, (B) The spectrum of tyrosine, (C) The spectrum of 3-nitrotyrosine. See section 2.2.1.4 for details of HPLC analysis.

3.2.2 Inhibition of acidic nitrite-mediated tyrosine nitration by procyanidins

In this section the ability of procyanidins to inhibit acidic nitrite-mediated tyrosine nitration was determined.

A. Catechin monomer

Exposure of tyrosine to acidic nitrite in the presence of catechin monomer inhibited the formation of 3-nitrotyrosine in a dose-dependent manner. IC_{50} of 3-nitrotyrosine formations by catechin monomer was found to be 51.9 μM (Fig 3.2). HPLC analysis showed that tyrosine was co-eluting with a broad peak, which remained unidentified (Fig 3.3A). One possibility is that these are the oxidation products of catechin upon interaction with nitrite in acid. At higher catechin monomer concentrations of 150 to 400 μM, total inhibition of tyrosine nitration was achieved, accompanied by the formation of two distinct peaks (Fig 3.3B).

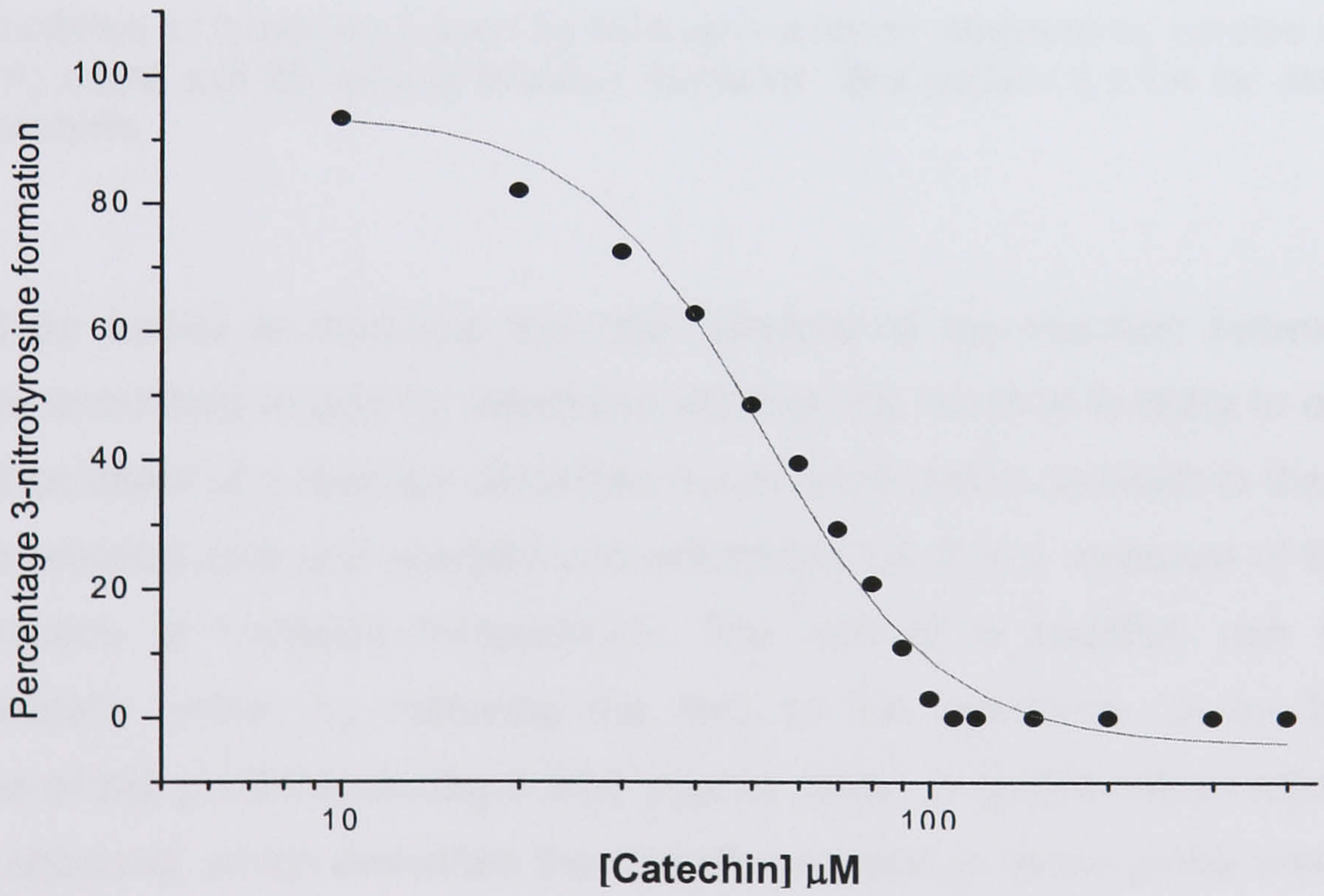


Fig 3.2 Inhibition of tyrosine nitration by catechin monomer. Catechin monomer (0 to 400 μM) was exposed to 400 μM nitrite in 0.5N HCl for 2 hours at 37°C to mimic the environment of the stomach. Results were obtained from 3 independent experiments and presented as mean ± SEM % inhibition that varied by less than 5.5%

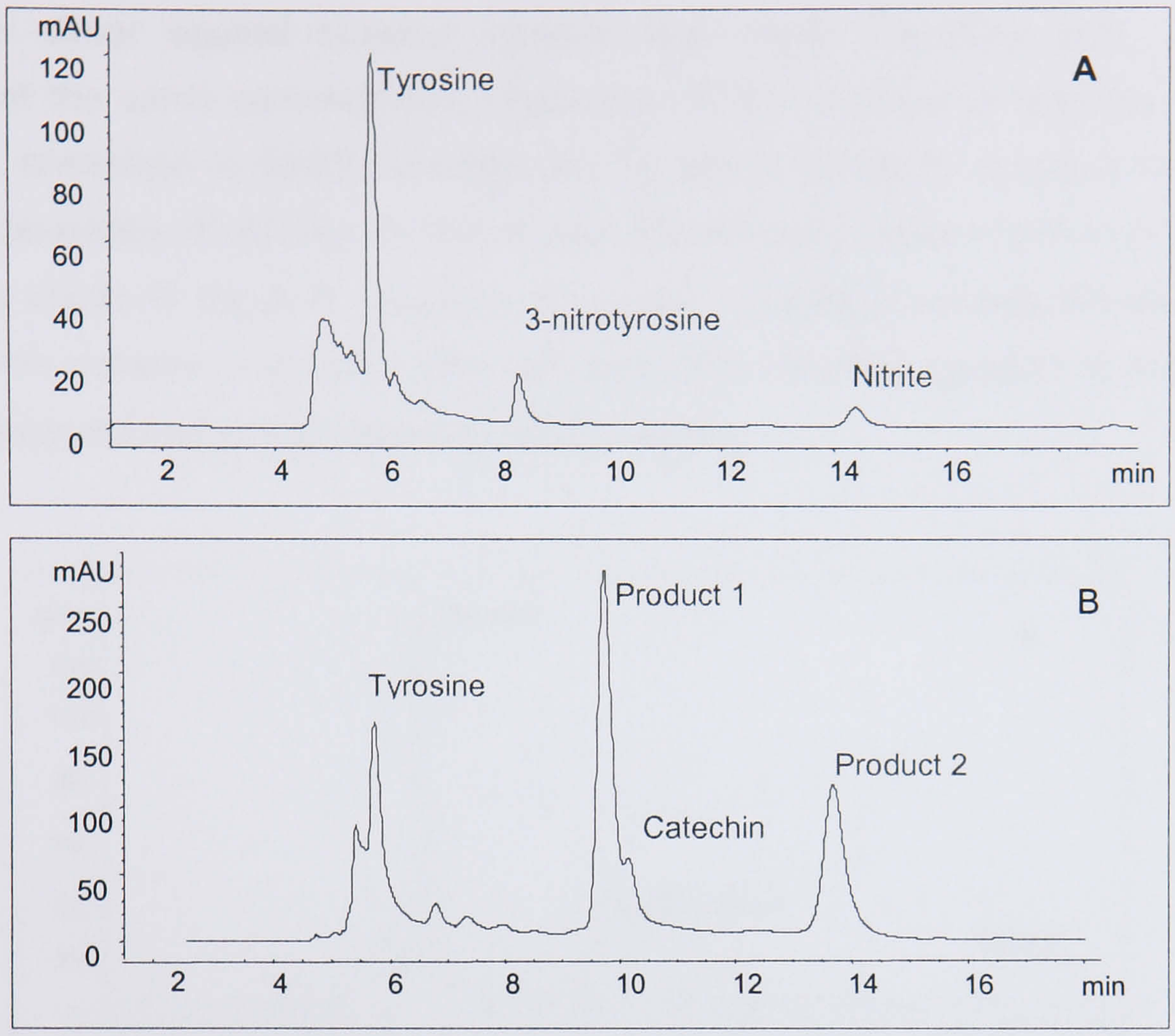


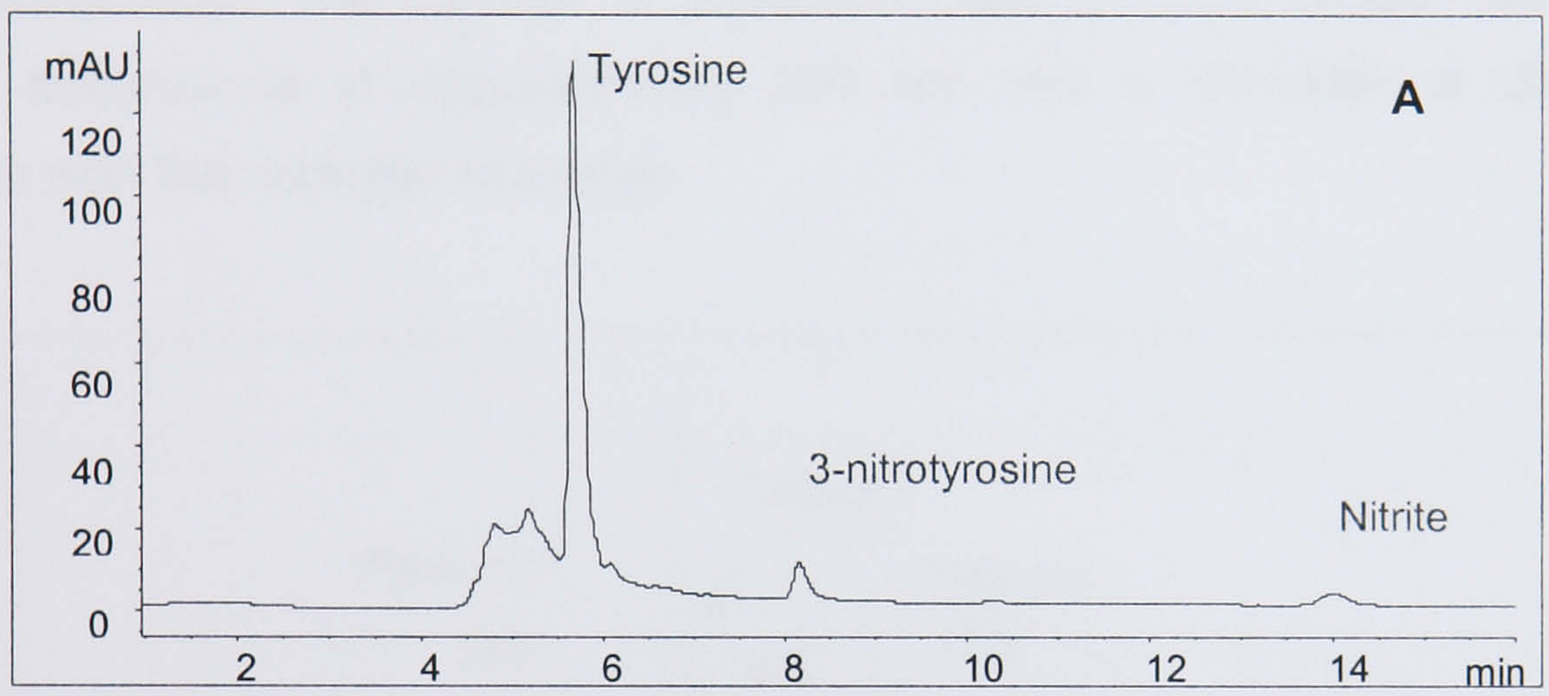
Fig 3.3 Inhibition of tyrosine nitration by catechin monomer analysed by reverse phase HPLC. (A) 50μM and (B) 400μM catechin monomer. See section 2.2.1.4 for details of HPLC analysis.

It would be useful to measure the rate constant of the reaction between catechin monomer and nitrite in acid to determine whether the reaction is likely to occur *in vivo*. The rate constant of a reaction describes the proportionality constant in the relationship between reaction rate and reactant concentrations i.e. It is a measure of the efficiency of a reaction at constant temperature. The rate of a reaction can be deduced experimentally, either by following the loss of the reactants, or by following the formation of the products during a time course study. A mathematical relationship can then be obtained, which describes the rate of a reaction in terms of the concentration of the reactant(s) multiplied by a constant, the rate constant.

B. Epicatechin dimer

A procyanidin dimer extract from cocoa beans containing two dimers was used for investigating the protective effects against acidic nitrite. The inhibition of 50 μM

epicatechin dimer against tyrosine nitration was more effectively than catechin monomer at the same concentration, displaying 66.9% inhibition of tyrosine nitration (Fig 3.4A), compared to 48.6% inhibition for the latter. Similar to catechin monomer, higher concentration of epicatechin dimer (400 μM) resulted in total inhibition of tyrosine nitration as shown in Fig 3.4B. However, the reaction profile of epicatechin dimer was different from catechin monomer, with only one major product (product I) and a few minor products formed at 400 μM of epicatechin dimer.



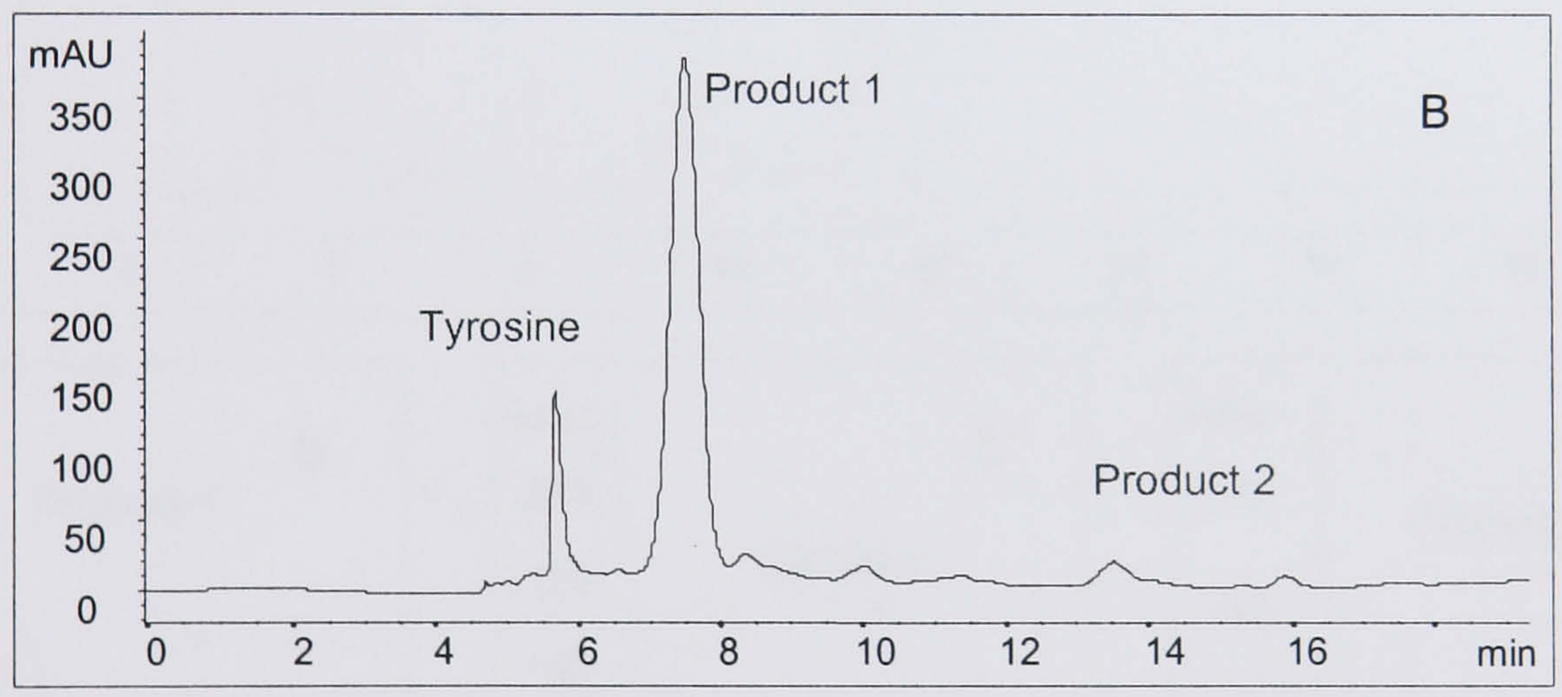


Fig 3.4 Inhibition of tyrosine nitration by epicatechin dimer analysed by reverse phase HPLC. (A) 50μM and (B) 400μM epicatechin dimer B2. See section 2.2.1.4 for details of HPLC analysis.

In order to identify these new products and determine the mechanism underlying the protection of procyanidins against tyrosine nitration, the direct interactions of procyanidins with acidic nitrite were investigated.

Interaction of flavonoids with acidic nitrite-generated reactive nitrogen species

3.2.3 Direct interaction of procyanidins and flavonoids with acidic nitrite

A. Catechin monomer

The direct interaction between catechin monomer and acidic nitrite yielded products with similar retention times and identical spectra as those observed in the inhibition study. Upon exposure of catechin monomer to acidic nitrite, two major products were formed, as shown in Fig 3.5A. The associated spectral characteristics of separated products I and II, in contrast to the native catechin monomer, are shown in Figs 3.5B, D and C, respectively. The spectra of products I and II were almost identical with increased absorbance at approximately 280 nm and a shoulder at 320 nm, in comparison with the catechin monomer.

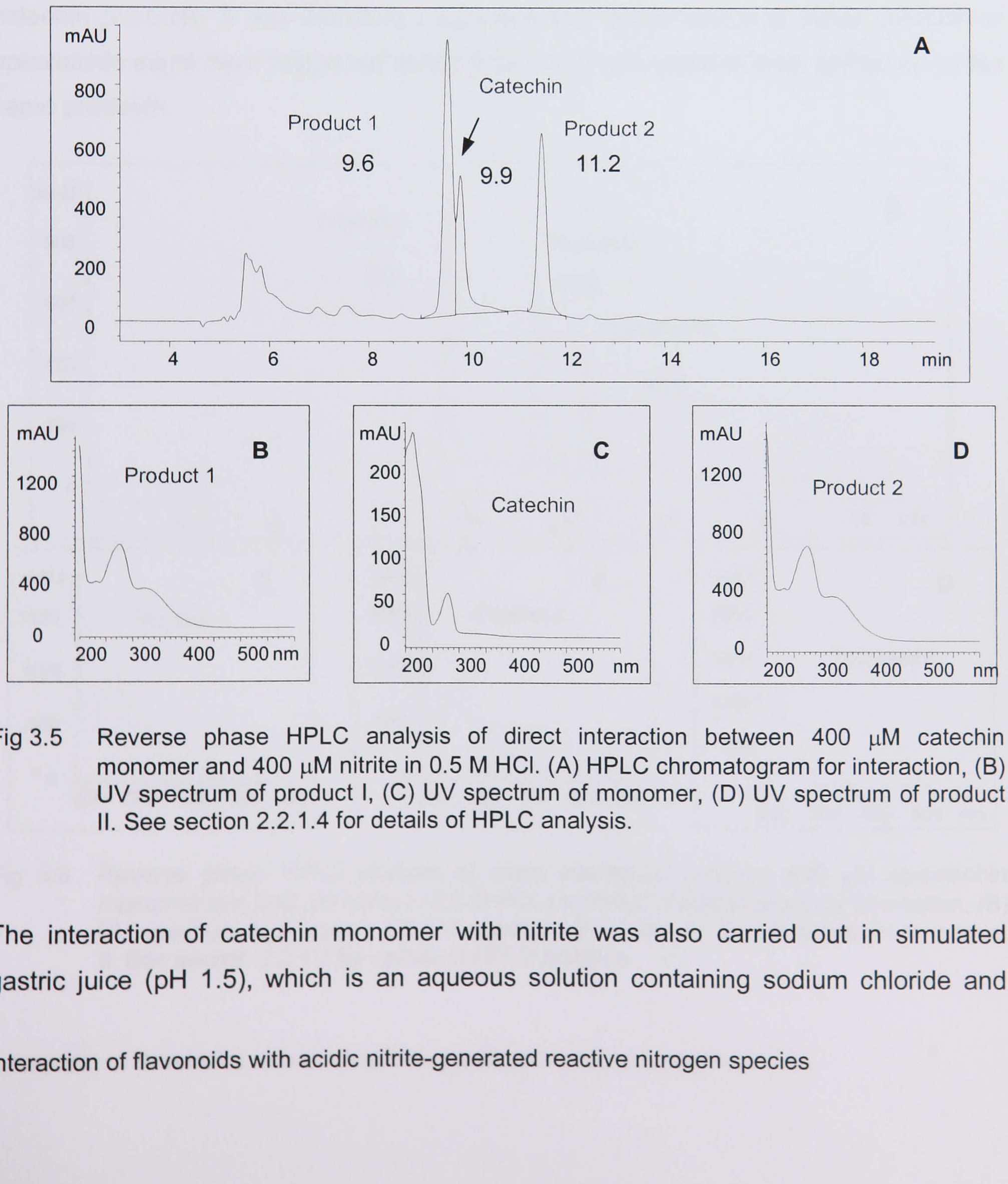


Fig 3.5 Reverse phase HPLC analysis of direct interaction between 400 μM catechin monomer and 400 μM nitrite in 0.5 M HCl. (A) HPLC chromatogram for interaction, (B) UV spectrum of product I, (C) UV spectrum of monomer, (D) UV spectrum of product II. See section 2.2.1.4 for details of HPLC analysis.

The interaction of catechin monomer with nitrite was also carried out in simulated gastric juice (pH 1.5), which is an aqueous solution containing sodium chloride and

HCl, but without pepsin. Similar results were obtained as in the acidic medium, with two main products formed having similar retention times as the products obtained in acid. Moreover, identical spectral characteristics such as increase in absorbance at 280 nm with appearance of a shoulder at 320 nm were obtained, further suggesting identical products were produced from the acidic medium and simulated gastric juice.

B. Epicatechin monomer

The direct interaction of epicatechin, an epimer of catechin, with acidic nitrite was also studied for comparison with catechin monomer. Epicatechin gave rise to two products with identical spectral characteristics as the products derived from catechin (Figs 3.6 A-D). Interestingly, the retention times of these products were also identical to the catechin products. It was therefore suggested that epimerisation of either catechin or epicatechin might have happened during interaction with nitrite in acid, giving rise to the same products.

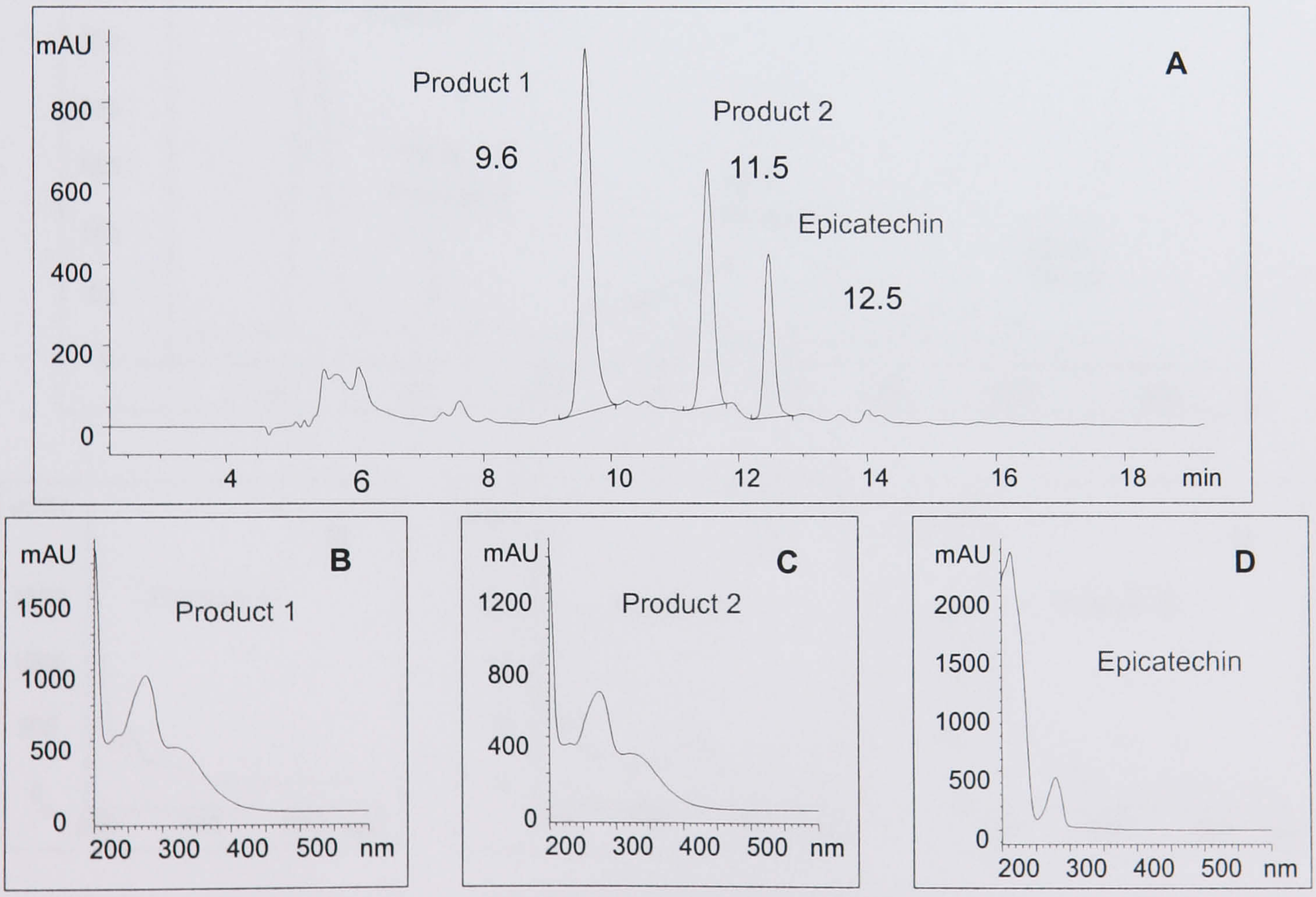


Fig 3.6 Reverse phase HPLC analysis of direct interaction between 400 μM epicatechin monomer and 400 μM nitrite in 0.5 M HCl. (A) HPLC chromatogram for interaction, (B) UV spectrum of product I, (C) UV spectrum of monomer, (D) UV spectrum of product II. See section 2.2.1.4 for details of HPLC analysis.

Interaction of flavonoids with acidic nitrite-generated reactive nitrogen species

C. Epicatechin Dimer

It was demonstrated in section 3.2.2 that upon inhibition of acidic nitrite-mediated tyrosine nitration by epicatechin dimer, one major and several minor products were produced. The direct interaction of epicatechin dimer was therefore undertaken and the products analysed by reverse phase HPLC to aid their identification. Figure 3.7A illustrates the HPLC chromatogram of the reaction and the products obtained. The associated spectrum of product 1 (Fig 3.7B) indicates an increase in absorbance at 280 nm with a shoulder at 320 nm, similar to the product formed in the interaction of acidic nitrite with catechin monomer. Apart from the major product, two minor products were also formed during the reaction between epicatechin dimer and acidic nitrite. Product II has a spectral characteristics typical of an anthocyanidin (Fig 3.7C), whereas the spectrum of product III was identical to that of product I (Fig 3.7D).

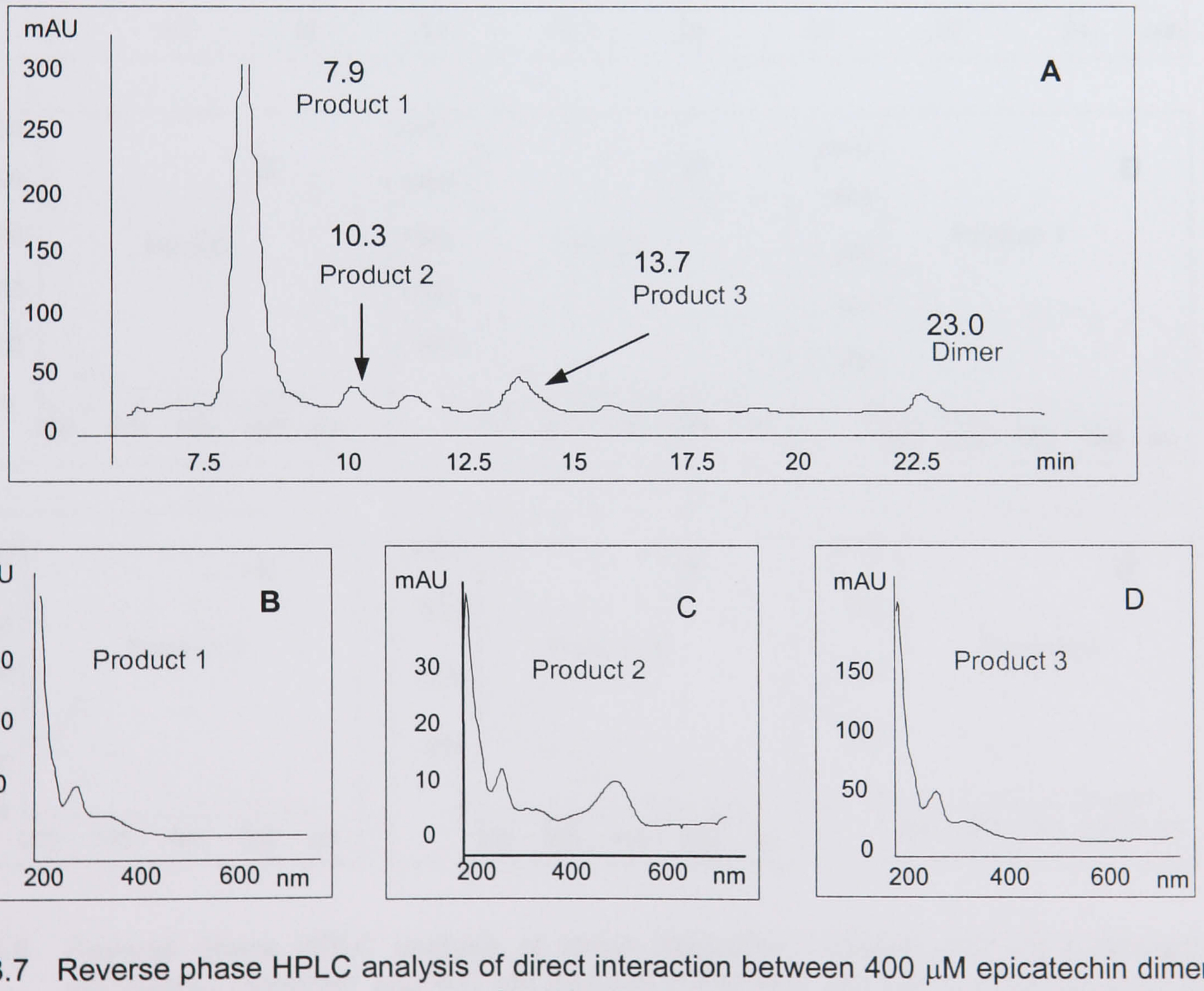


Fig. 3.7 Reverse phase HPLC analysis of direct interaction between 400 μM epicatechin dimer and 400 μM nitrite in 0.5 M HCl. (A) HPLC chromatogram for interaction, (B) UV spectrum of product I, (C) UV spectrum of product II, (D) UV spectrum of product III. See section 2.2.1.4 for details of HPLC analysis.

D. Methylated Epicatechin

The structure of methylated epicatechins (Me-ECs) differs from epicatechin by a substitution of one methyl group on the catechol structure of the B ring (Fig 1.7). In order to understand the role of the catechol structure upon interaction with acidic nitrite, the direct interaction of Me-EC with acidic nitrite was undertaken.

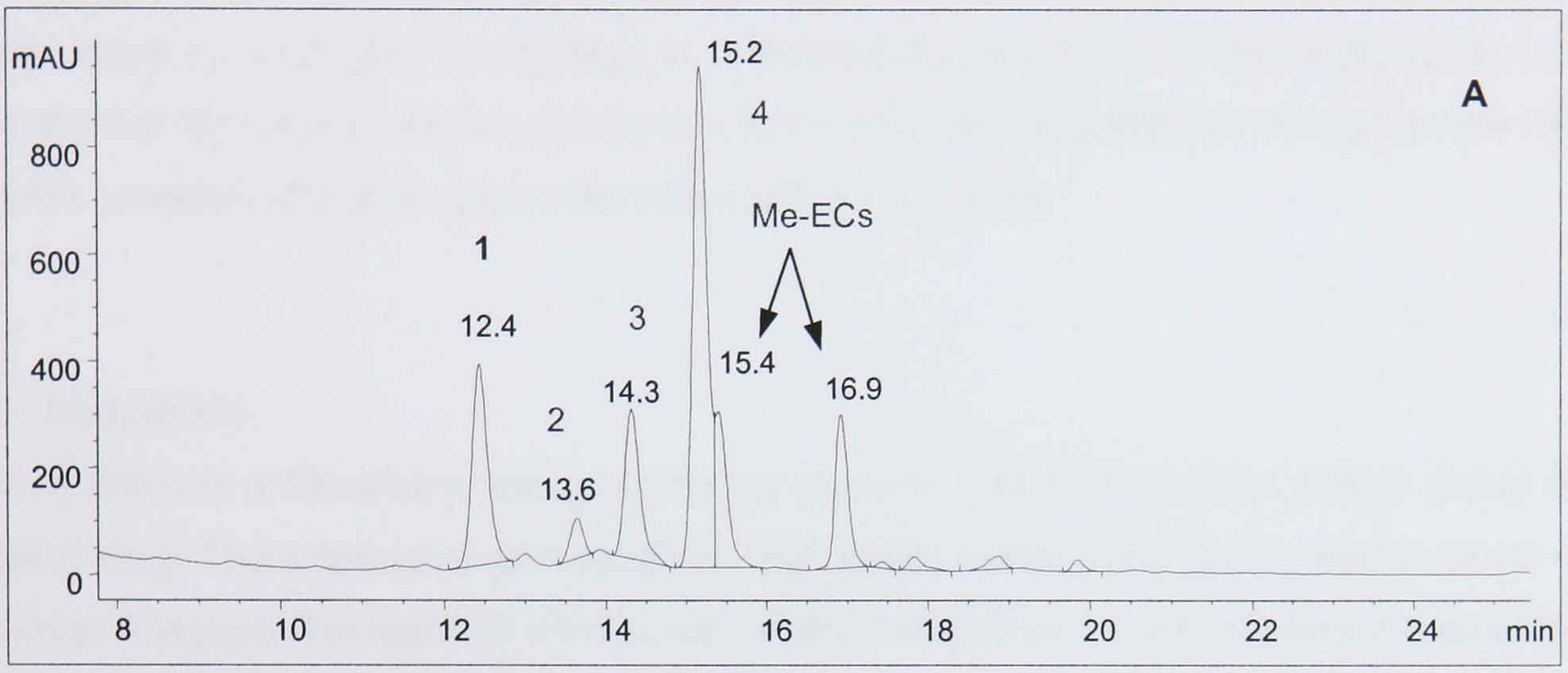


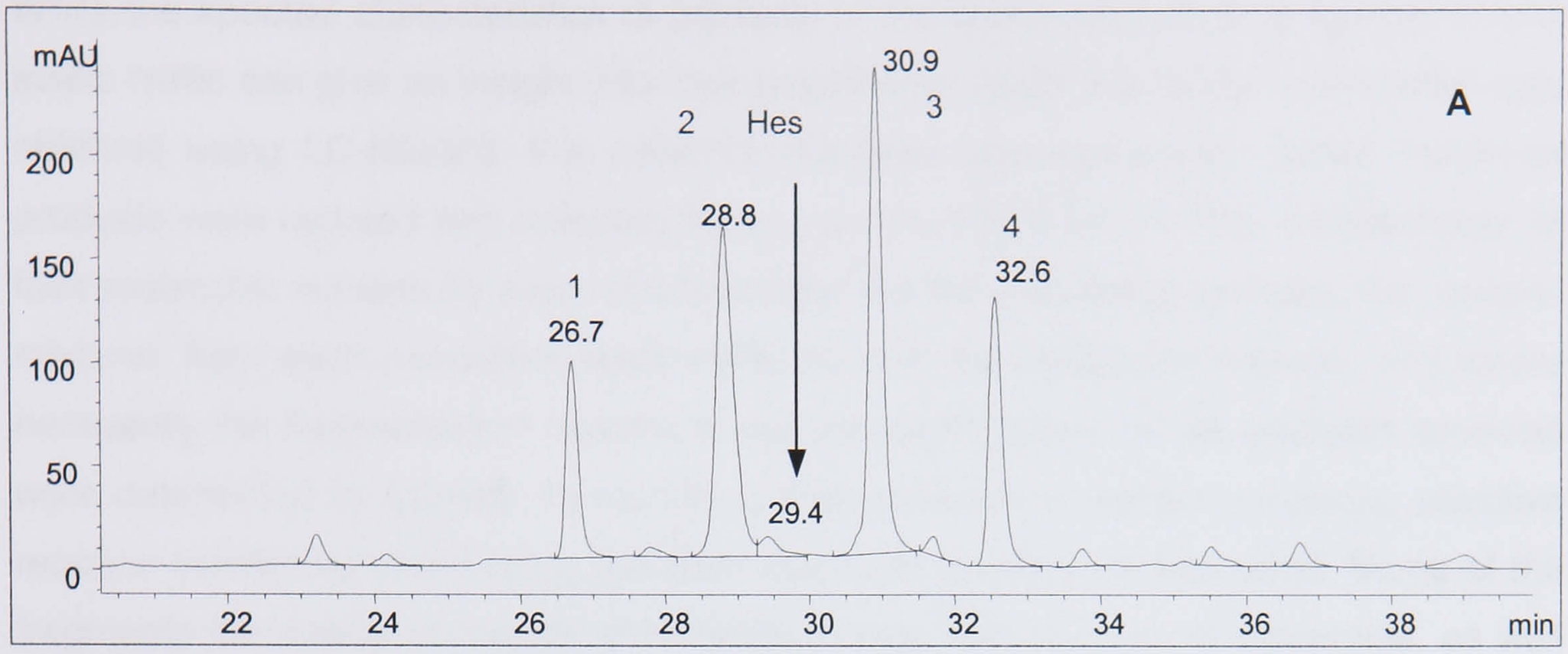
Fig 3.8 Reverse phase HPLC analysis of direct interaction between 400 μM methylated epicatechin monomer and 400 μM nitrite in 0.5 M HCl. (A) HPLC chromatogram for interaction, (B) UV spectrum of 3′-Me-EC, (C) UV spectrum of 4′-Me-EC, (D) UV spectrum of product I, (E) UV spectrum of product 2, (F) UV spectrum of product 3, (G) UV spectrum of product 4. See section 2.2.1.4 for details of HPLC analysis.

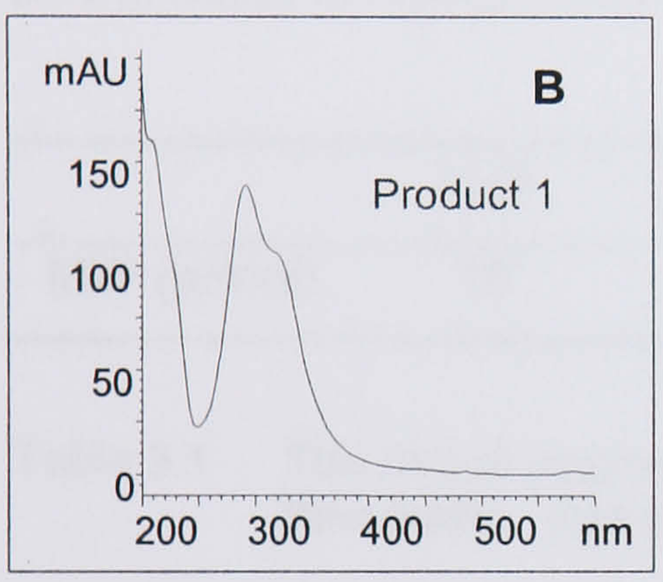
A mixture of 3′ and 4′ Me-ECs was used for the experiments and their retention times were approximately 15.4 and 16.9 min, respectively (Fig 3.8A). The spectra of Me-ECs were similar to that of epicatechin, as shown in Figs 3.8B&C. After incubation with acidic nitrite for 2h four distinct peaks were formed which all have similar spectral characteristic as the products derived from epicatechin (Fig 3.8D-G). Further analysis with mass spectrometry is required to determine the identities of these products, and hence the reaction profile of epicatechin and methylated epicatechin as well as the role of the catechol structure upon interaction with acidic nitrite.

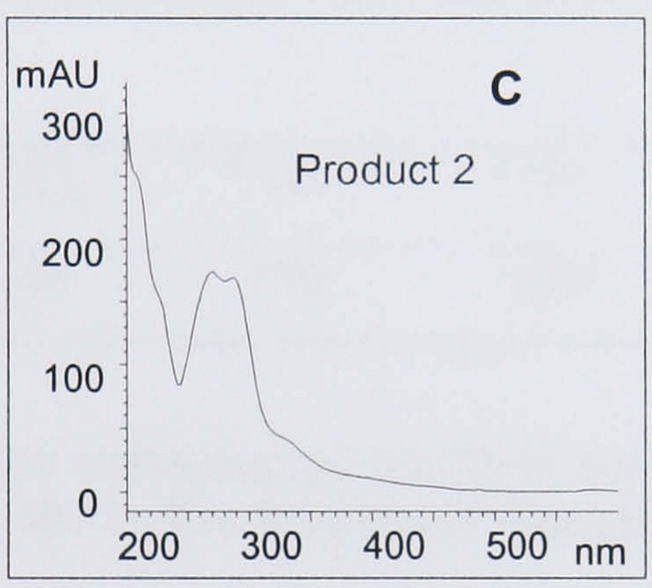
E. Hesperetin

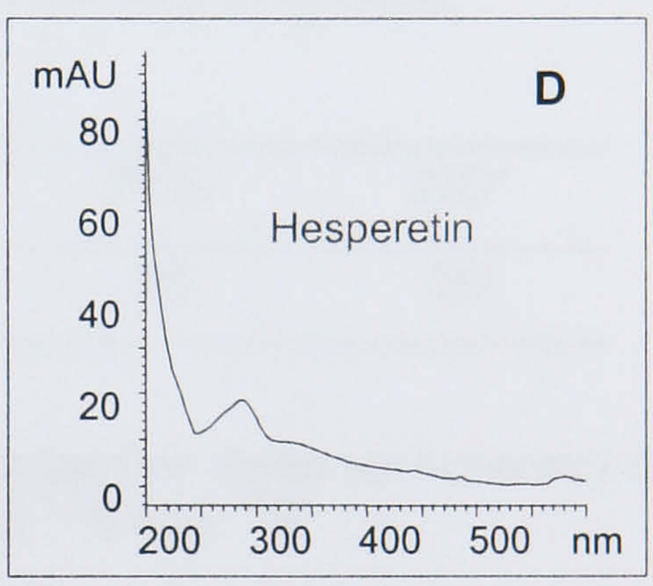
Hesperetin is a flavanone with a 4′-methyl group on the B ring and a 4-keto group on the C ring. The interaction of hesperetin with acidic nitrite was investigated in order to further compare the reaction mechanism of flavonoids that do not possess the catechol group with nitrite in acid.

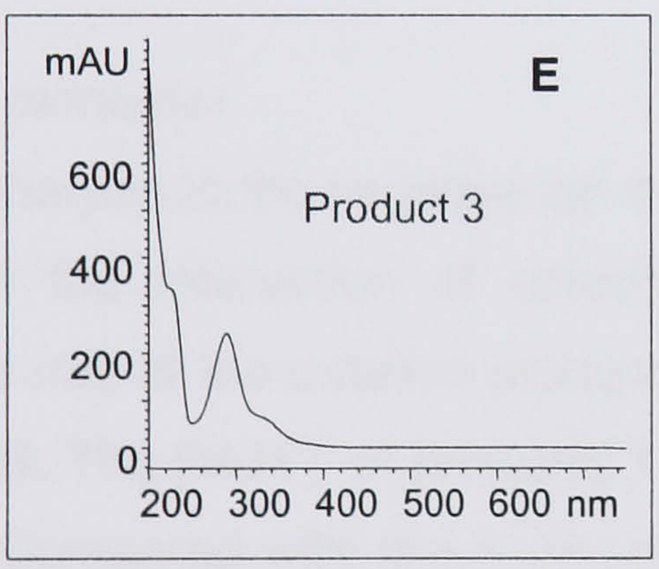
Figure 3.9 depicts the interaction of 100 μM hesperetin with 400 μM nitrite in acid. Four major products were formed each had very distinct spectral characteristics. Unlike the flavanols (Arteel *et al.,* 2000, Oldreive *et al.,* 1998, Pannala *et al.,* 1997), the interaction of hesperetin with RNS has not been as well studied, and knowledge of the reaction mechanism as well as the spectral characteristics of the products is not very well understood. It is therefore difficult to identify the products based on their UV spectrum. Further analysis such as mass spectrometry is required to aid determination of their identities and thus the reaction mechanism of hesperetin with acidic nitrite.











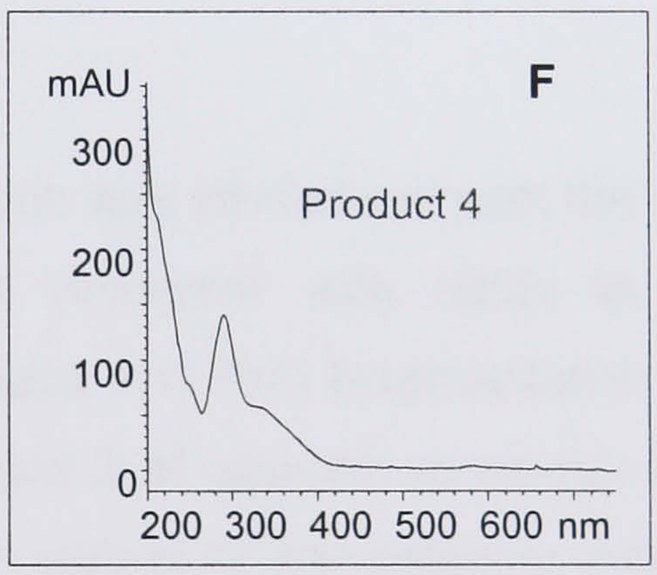


Fig 3.9 Reverse phase HPLC analysis of direct interaction between 400 μM hesperetin and 400 μM nitrite in 0.5 M HCl. (A) HPLC chromatogram for interaction, (B) UV spectrum of product 1, (C) UV spectrum of product 2, (D) UV spectrum of hesperetin, (E) UV spectrum of product 3, (F) UV spectrum of product 4. See section 2.2.1.4 for details of HPLC analysis.

3.2.4 LC-MS/MS analysis

While the spectral characteristics of products of the direct interaction of flavonoids and acidic nitrite can give an insight into their possible structure, structural confirmation was obtained using LC-MS/MS. For catechin monomer and epicatechin dimer, individual products were isolated and collected by preparative HPLC prior to the determination of their molecular masses by mass spectrometry. For the remaining samples, the reaction mixtures from each interaction were collected and the molecular masses, and where necessary the fragmentation spectra to aid the identification, of all unknown products were determined by LC-MS. To identify certain products in reaction mixtures, selective reaction monitoring (SRM) using the most abundant product ion was used. Some of the fragments commonly encountered in tandem mass spectrometry of flavonoids, as well as the mass to charge ratio (*m/z)* of some RNS are listed in the following table.

	H_2O	CO_2	$^{1,3}A^{+}$ *	$^{1,2}B^{+}$ *	NO_2^-	$NO^{\bullet}$
MW (g/mol)	18	44	139	123	46	30

Table 3.1 The *m/z* of fragments commonly encountered during tandem mass spectrometry of flavonoids, * See p. 121 for the structure of fragments $^{1,3}A^{+}$and $^{1,3}B^{+}$.

A. Catechin monomer

LC-MS/MS analysis in the negative ion mode was carried out with the isolated products derived from the interaction of catechin monomer with nitrite in acid. Table 3.2 illustrates the *m/z* of the putative product ions and their fragmentation pattern obtained by LC-MS/MS. The $[M\text{-}H^{+}]^{-}$ of products 1 and 2 of catechin monomer have an identical *m/z* of 347. Compared with the molecular weight of the catechin monomer (290 Da), both products have an increased molecular weight of 58 Da. Taking into account the required loss of a hydrogen during the reaction, both products were suggested to be dinitrosation products of the catechin monomer ($+NO^{\bullet}\text{-}H$ = 29 Da). This was confirmed by their fragmentation patterns, which were identical and therefore indicate that they are likely to have similar structural features. The first fragmentation of both products resulted in a loss of a structure with *m/z* of 36 (Table 3.2), possibly two water units. The interaction between catechin with nitrite in acid was also analysed in the positive ion mode (see section 2.2.1.6 for the choice of ion polarity).

m/z of products & fragmentation products	*Products of catechin monomer*		*Products of epicatechin dimer*		
	1	*2*	*1*	*2*	*3*
Initial products	347	347	635	577	606
Fragmentation 1	311	311	483	425	454
Fragmentation 2	-	-	465	407	436
Fragmentation 3	-	-	313	-	255

Table 3.2 $[M-H^+]^-$ ions of products derived from the direct interaction of procyanidins with nitrite in simulated gastric juice, and their fragmentation pattern. All spectra were recorded in negative ion mode except that of the epicatechin dimer product 2 which was recorded in the positive ion mode.

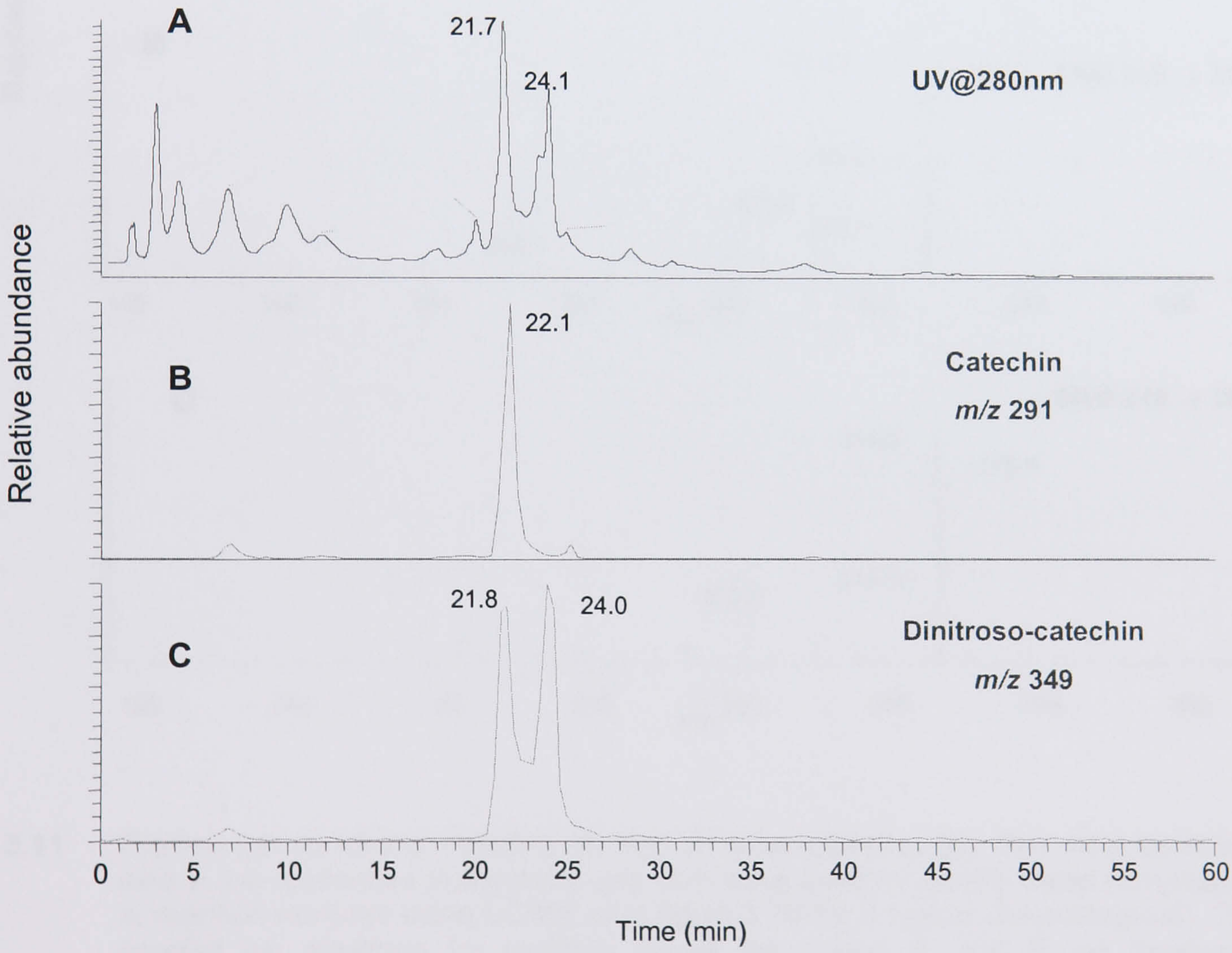


Fig 3.10 LC-MS/MS analysis of the reaction mixture from interaction between catechin and nitrite in acid. All spectra were recorded in the positive ion mode. The figure shows combined UV (280nm, top trace) and MS traces. Catechin and dinitroso-catechin were detected using typical fragmentation reactions for these compounds, i.e. 291 → 139 for catechin (fig 3.11A) and 349 → 315 for the dinitroso catechin (fig 3.11B).

Interaction of flavonoids with acidic nitrite-generated reactive nitrogen species

The LC-MS/MS chromatograms and product ion spectrum of a dinitroso-catechin are illustrated in Fig 3.10 and 3.11. The two major products detected have identical *m/z* of 349 (positive ion mode) and a major fragment ion with an *m/z* of 314 and 315 (Fig 3.11B for product 1 and 3.11C for product 2). As such they were concluded to be isomers of dinitroso-catechin.

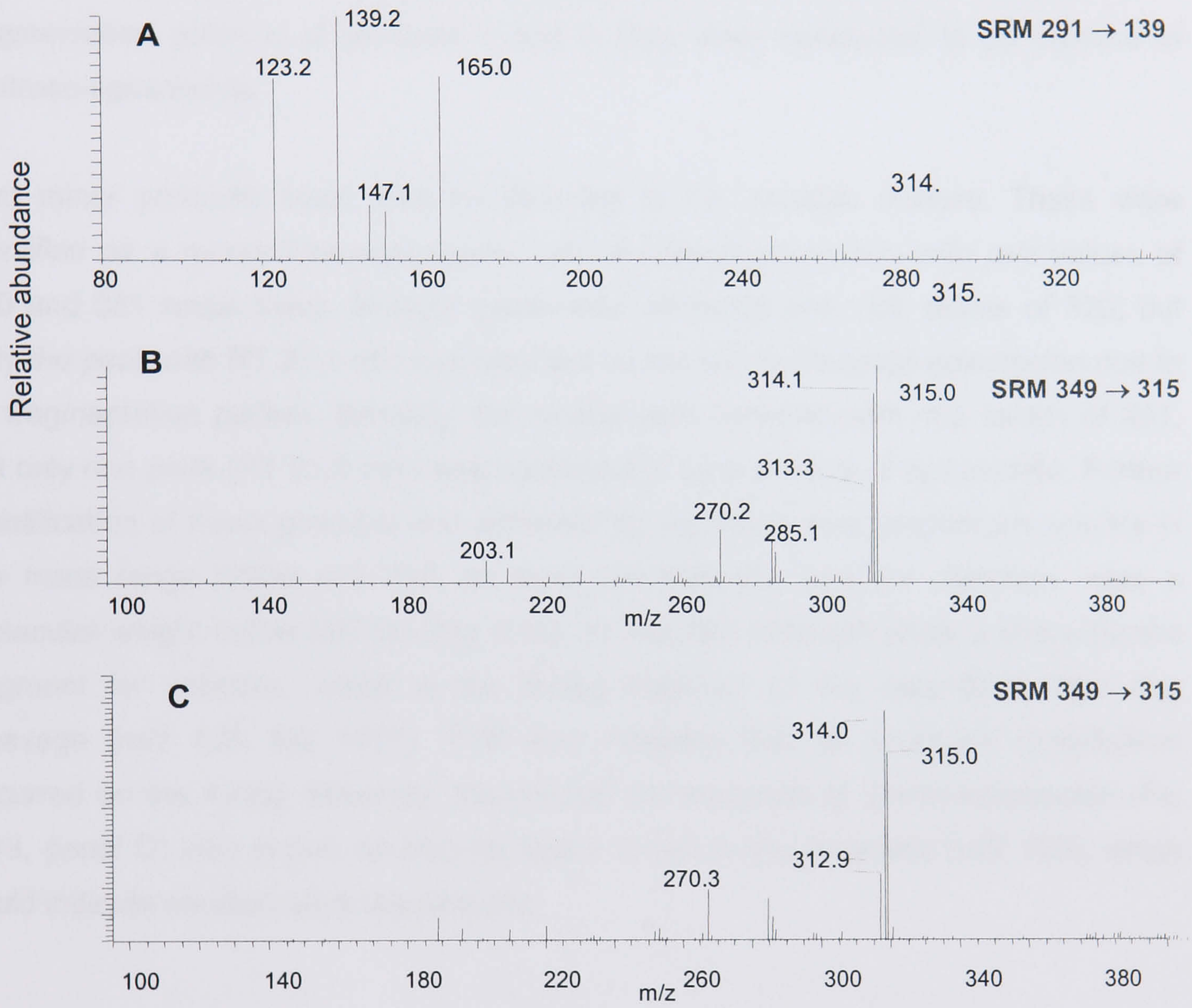


Fig 3.11 Product ion spectra of catechin (A) and dinitroso-catechin (B). The most abundant ions in the respective product ion spectrum were used to identify these compounds in reaction mixtures using LC/MS (see figure 3.10 for a typical chromatogram). The product ion spectrum for catechin shows the typical A- and B-ring fragments resulting from a retro-Diels-Alder reaction.

B. Epicatechin monomer

To further identify the reaction products of epicatechin monomer with nitrite, LC-MS/MS was carried out with the reaction mixture (Fig 3.12). Similar to catechin monomer, the two major products detected have identical *m/z* of 349 (positive ion mode) and a major fragment ion with an *m/z* of 314 and 315 (Fig 3.12C, only one spectrum is shown but both products resulted in the same fragmentation), suggesting these were epicatechin that had undergone di-nitrosation. Because of the identical *m/z* values and fragmentation patterns of products 1 and 2, they were concluded to be isomers of dinitroso-epicatechin.

Two minor products could also be detected in the reaction mixture. These were identified as a mononitroso-epicatechin and a dinitro-epicatechin, with *m/z* values of 320 and 381 respectively. Multiple peaks were detected with *m/z* values of 320, but only the peak with RT 27.1 min was detected as the mononitrosated epicatechin due to its fragmentation pattern. Similarly, two peaks were detected with *m/z* values of 381, but only one peak (RT 21.6 min) was confirmed to be a product of epicatechin. Further identification of these products was achieved by recording their product ion spectra in the mass range below *m/z* 210, as most characteristic ions for catechins have a molecular weight below 200 Da (Fig 3.13). All reaction products show a characteristic fragment for catechin, which is the B-ring fragment of the retro-Diels-Alder ring cleavage (*m/z* 123, Ma 1997). This also indicates that all structural modification occurred on the A-ring. However, the product ion spectrum of dinitro-epicatechin (Fig 3.13, panel D) also shows an intense signal for an A-ring fragment (*m/z* 139), which could indicate an alternative reaction site.

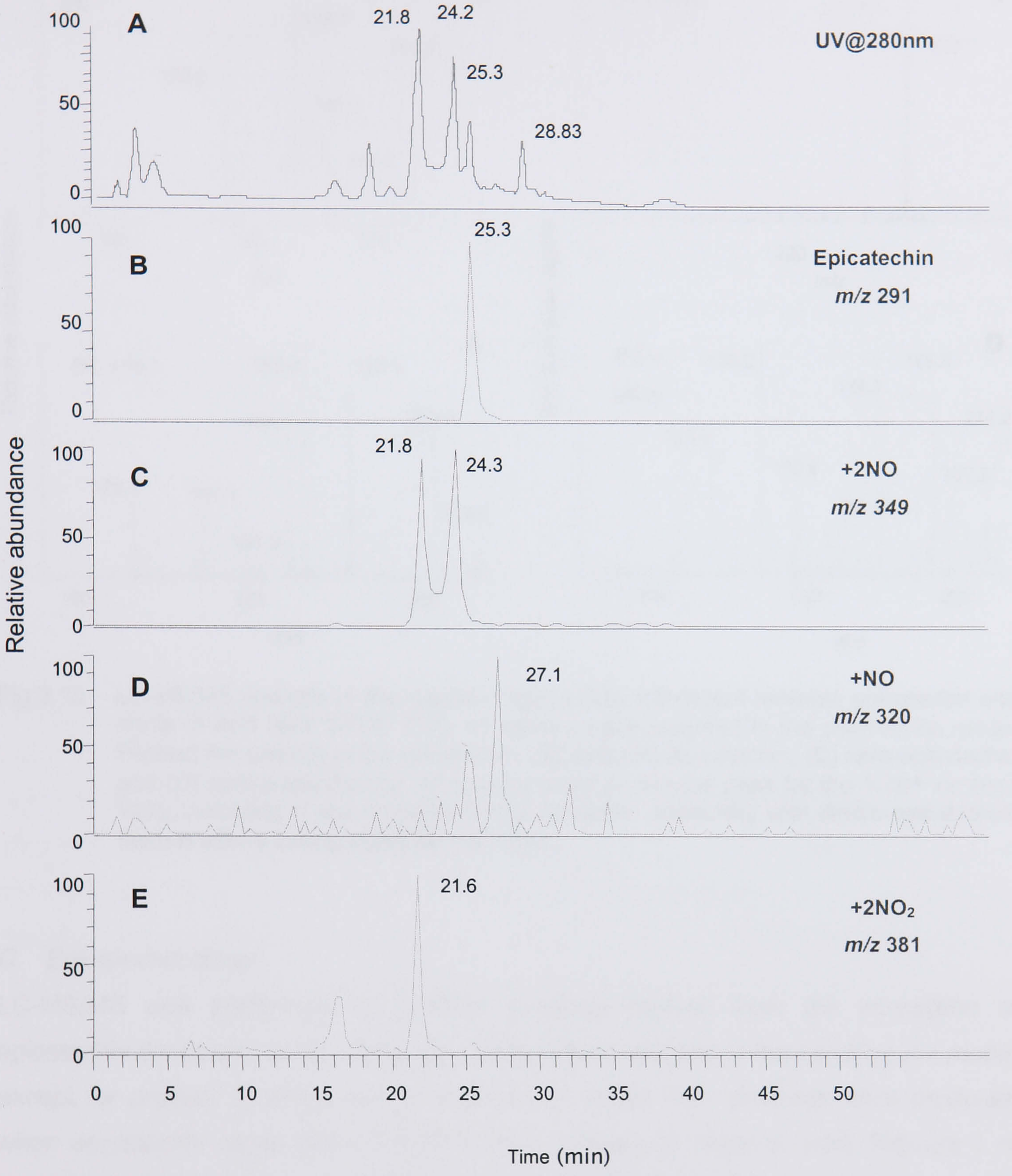


Fig 3.12 LC-MS/MS analysis of the reaction mixture from interaction between epicatechin and nitrite in acid. The figure shows combined UV (280nm, top trace) and MS traces. Epicatechin and dinitroso-epicatechin were detected using typical fragmentation reactions for these compounds, ie 291 → 139 for epicatechin, 349 → 314, 315 for the dinitroso epicatechin, 320 → 123 for nitroso-epicatechin and 381 → 123 for the dinitro-epicatechin. The product ion spectra are shown in Fig 3.13.

Interaction of flavonoids with acidic nitrite-generated reactive nitrogen species

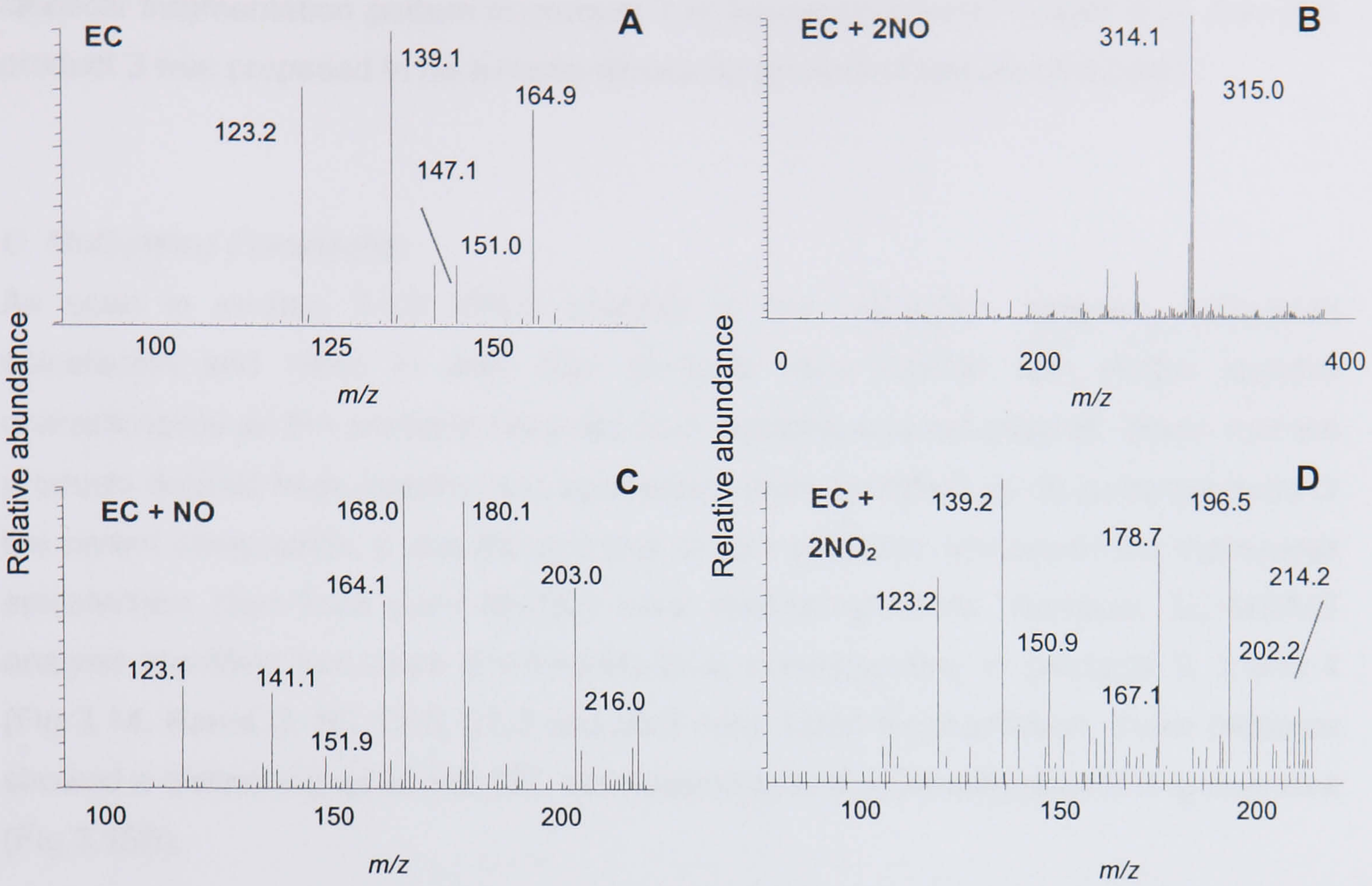


Fig 3.13 LC-MS/MS analysis of the reaction mixture from interaction between epicatechin and nitrite in acid (m/z 100 to 210). All spectra were recorded in the positive ion mode. Product ion spectra of (A) epicatechin; (B) dinitroso-epicatechin; (C) nitro-epicatechin and (D) dinitro-epicatechin. All spectra show an intense peak for the $^{1,2}B^{+}$ ion (m/z 123), indicating a non-modified B-ring. However, especially with dinitro-epicatechin, there is also a strong signal for the $^{1,3}A^{+}$.

C. Epicatechin dimer

LC-MS/MS was performed on isolated products derived from the interaction of epicatechin dimer and acidic nitrite. All spectra were recorded in the negative ion mode, except for product II, which was in positive ion mode. Two products were produced when epicatechin dimer ($[M\text{-}H^{+}]^{-}$ = 577) was exposed to nitrite in acid. Product 1 of epicatechin dimer had a *m/z* of 635, corresponding to the addition of a structure with molecular weight of 58 Da, suggesting the addition of two nitroso groups. The first fragmentation of this product gave rise to a new structure with *m/z* of 483 (loss of 152), and subsequent fragmentations resulted in a loss of water (*m/z* 18), followed by another loss of 152 (Table 3.2). The *m/z* of product 2 (positive ion mode) and product 3 were 577 and 606, respectively. Identification of product 2 proved to be slightly difficult since the molecular weight of 578 does not correspond to any product. The *m/z* of product 3 indicates an addition of a structure with molecular weight of 30, with an

identical fragmentation pattern to product 1 of epicatechin dimer (Table 3.2). As such, product 3 was proposed to be a mono-nitrosated product of epicatechin dimer.

D. Methylated Epicatechin

As seen in section 3.2.3 HPLC analysis of the interaction between methylated epicatechin and nitrite in acid, four products were formed with similar spectral characteristics as the products obtained from catechin and epicatechin. Given that the products derived from catechin and epicatechin were identified as dinitroso-products of the parent compounds, it was thought that all four products produced from methylated epicatechins (two from each Me-EC) were dinitroso-products. However, LC-MS/MS analysis identified just three dinitroso-Me-ECs, corresponding to products 2, 3 and 4 (Fig 3.14, Panel D, RT 25.5, 27.3 and 28.3 min). Upon fragmentation, these products showed a distinct signal at *m/z* 137, corresponding to the *O*-methylated B ring fragment (Fig 3.15B).

Owing to the spectral characteristics similarity of product 1 to products 2,3 and 4, this was thought to be similar in structure to the other products. However, no information was obtained on the molecular weight of product 1 and thus there was no evidence to suggest it was a dinitroso product. LC-MS/MS also suggested the presence of a mono-nitrated product in the reaction mixture, (Fig 3.14C, RT 30.3 min), and the corresponding product ions spectra in Fig 3.15A. In addition, at least three dinitrated products were also detected in the reaction mixture (Fig 3.14E, RT 24.2, 27.4 and 28.7 min), and the corresponding product ions spectrum for one of the products is illustrated in Fig 3.15A. Me-EC has five possible sites for NO_2 addition, resulting in the possibility for more than two isomers. All products show a significant signal at *m/z* 305, corresponding to methylated epicatechin and suggesting the loss of the modifying group. Although several other peaks were detected with *m/z* 395, it was not possible to identify their structures from the product ion scans, and they were thought to be interference compounds as none showed a signal for *m/z* 305.

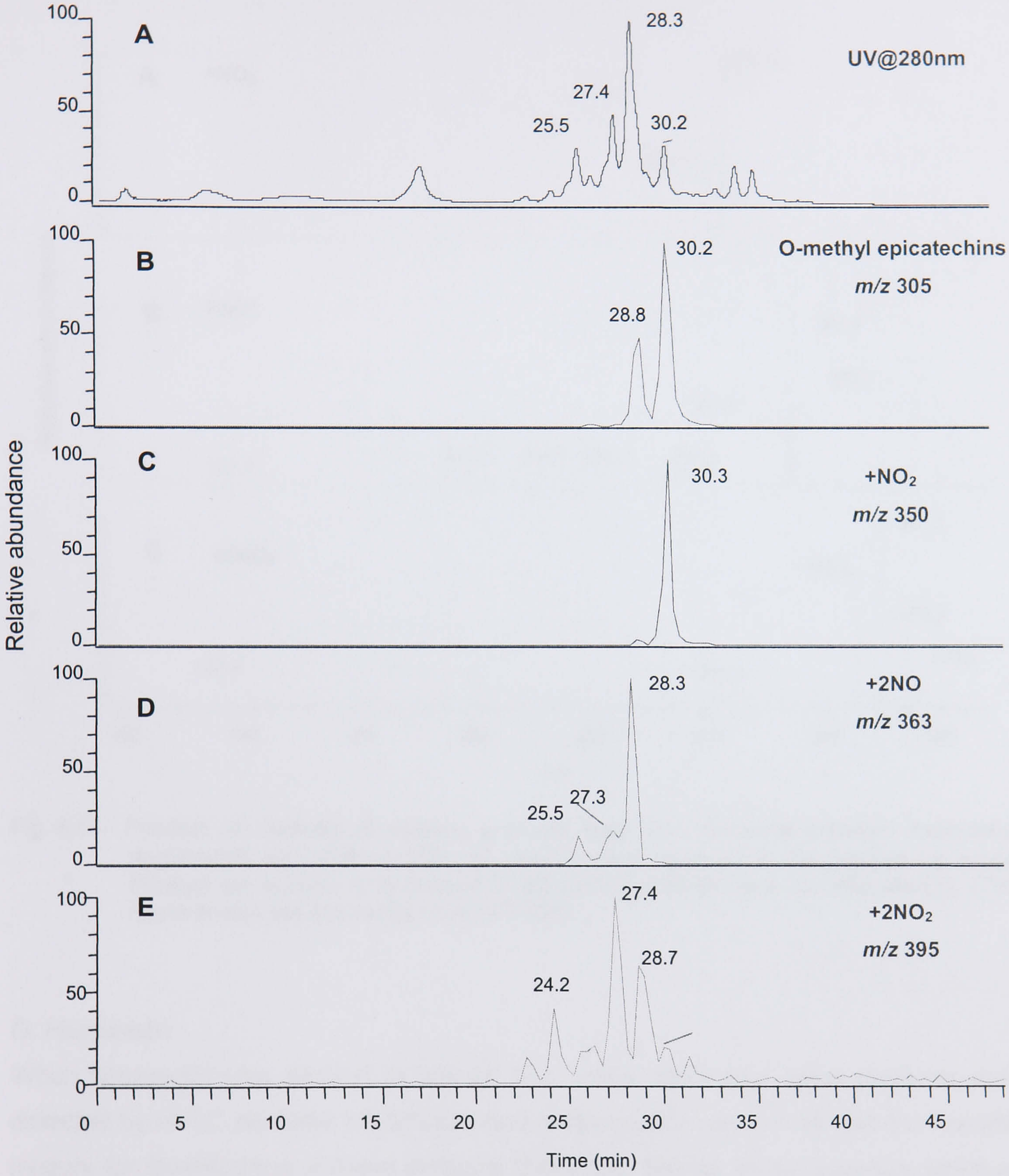


Fig 3.14 LC-MS/MS analysis of the reaction mixture from interaction between methylated epicatechins and nitrite in acid. All spectra were recorded in the positive ion mode. This figure shows the combined UV and SRM traces for the analysis of the reaction mixture. All compounds were detected by a typical fragmentation reaction, i.e. 350 → 305 for nitro-Me-EC and 395 → 305 for dinitro-Me-ECs. The product ion spectra for the nitrated and nitrosated compounds are shown in Fig. 3.15.

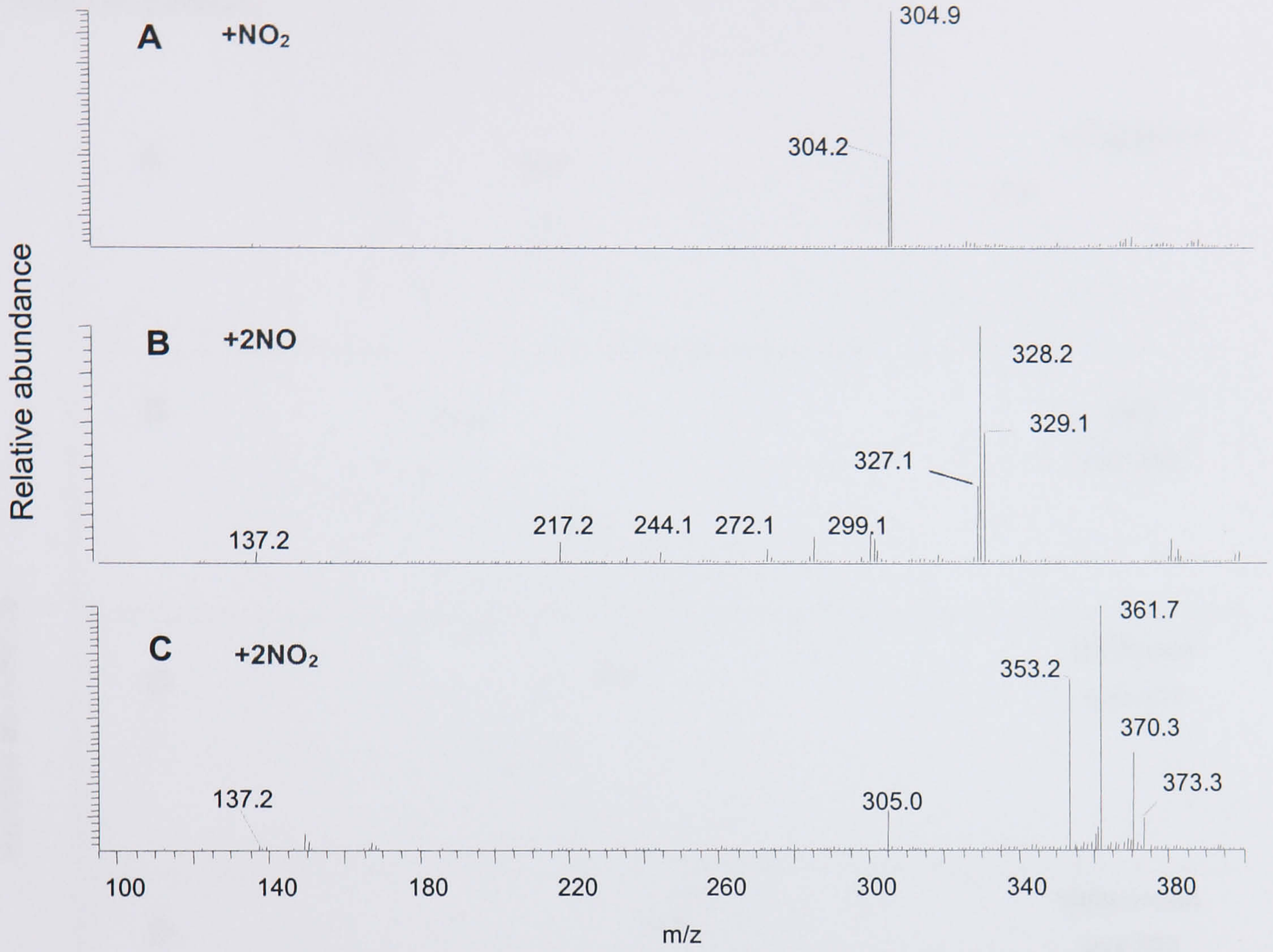


Fig 3.15 Product ion spectra of reaction products from the interaction between methylated epicatechin and nitrite in acid. All spectra were recorded in the positive ion mode. Product ion spectra of nitro-Me-EC; (B) dinitroso-Me-EC and (C) nitro-Me-EC. (This figure shows the m/z range from 100-400).

D. Hesperetin

When hesperetin was allowed to interact with acidic nitrite four major products were detected by HPLC analysis. LC-MS/MS was subsequently carried out with the reaction mixture for identification of these products (Fig 3.9A). Similar to the flavanols which all gave rise to nitrosated products, the first product of hesperetin showed an abundant signal at *m/z* 332, which was identified as a mono-nitroso hesperetin (Fig 3.16B). LC-MS/MS revealed the *m/z* of product 2 for $[M+H^+]^+$ was 317, which remained unidentified (Fig 3.16C). Both products 3 and 4 showed strong signals at *m/z* for $[M+H^+]^+$ 348, corresponding to addition of 45 in molecular weight over hesperetin (Fig 3.16E, RT 49.1 and 50.9 min). This suggested both products 3 and 4 had substitution of one nitro

group (MW = 46, minus H^+) on their structure, resulting in their identification as isomers of nitro-hesperetin.

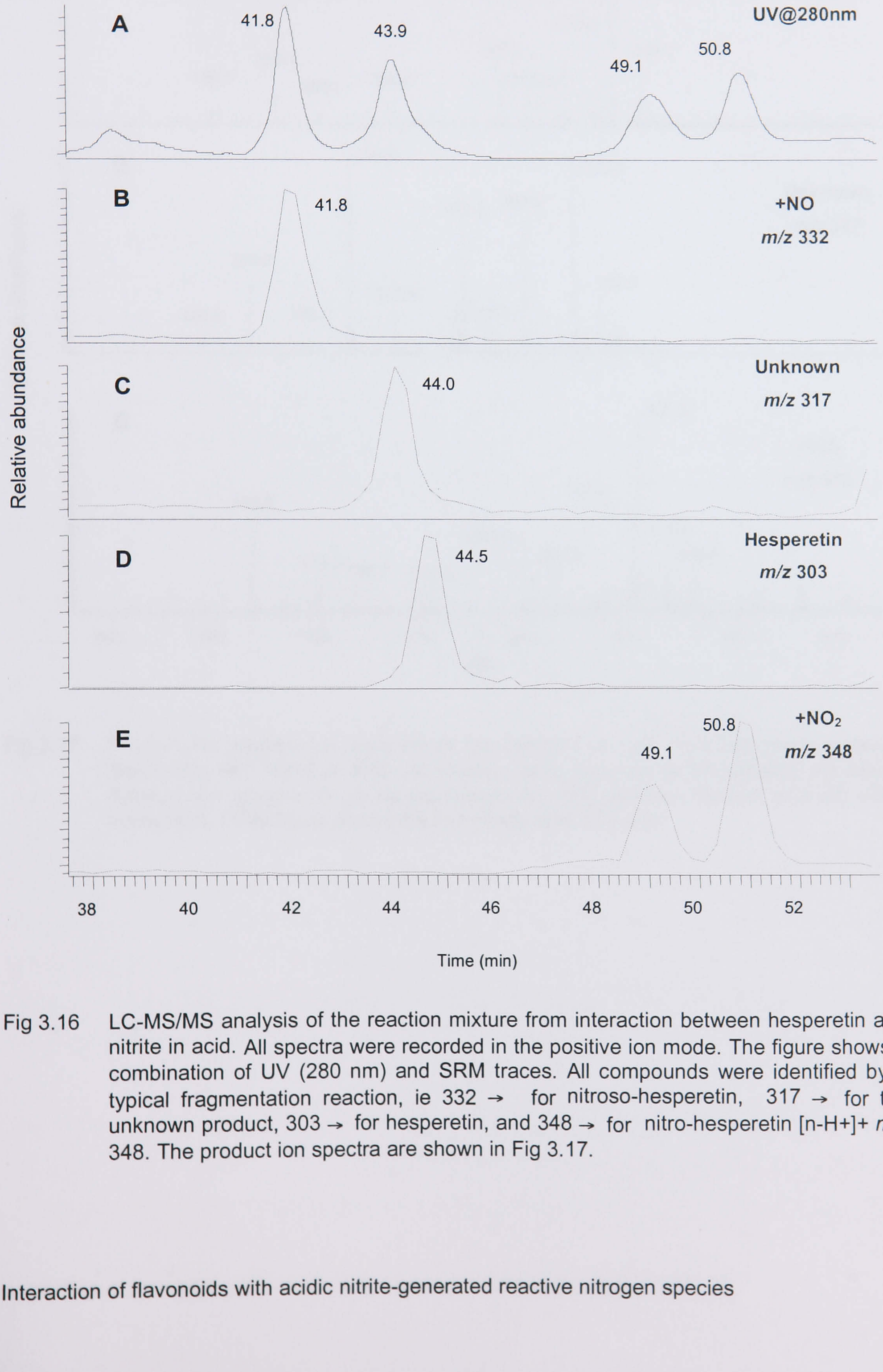


Fig 3.16 LC-MS/MS analysis of the reaction mixture from interaction between hesperetin and nitrite in acid. All spectra were recorded in the positive ion mode. The figure shows a combination of UV (280 nm) and SRM traces. All compounds were identified by a typical fragmentation reaction, ie 332 → for nitroso-hesperetin, 317 → for the unknown product, 303 → for hesperetin, and 348 → for nitro-hesperetin [n-H+]+ *m/z* 348. The product ion spectra are shown in Fig 3.17.

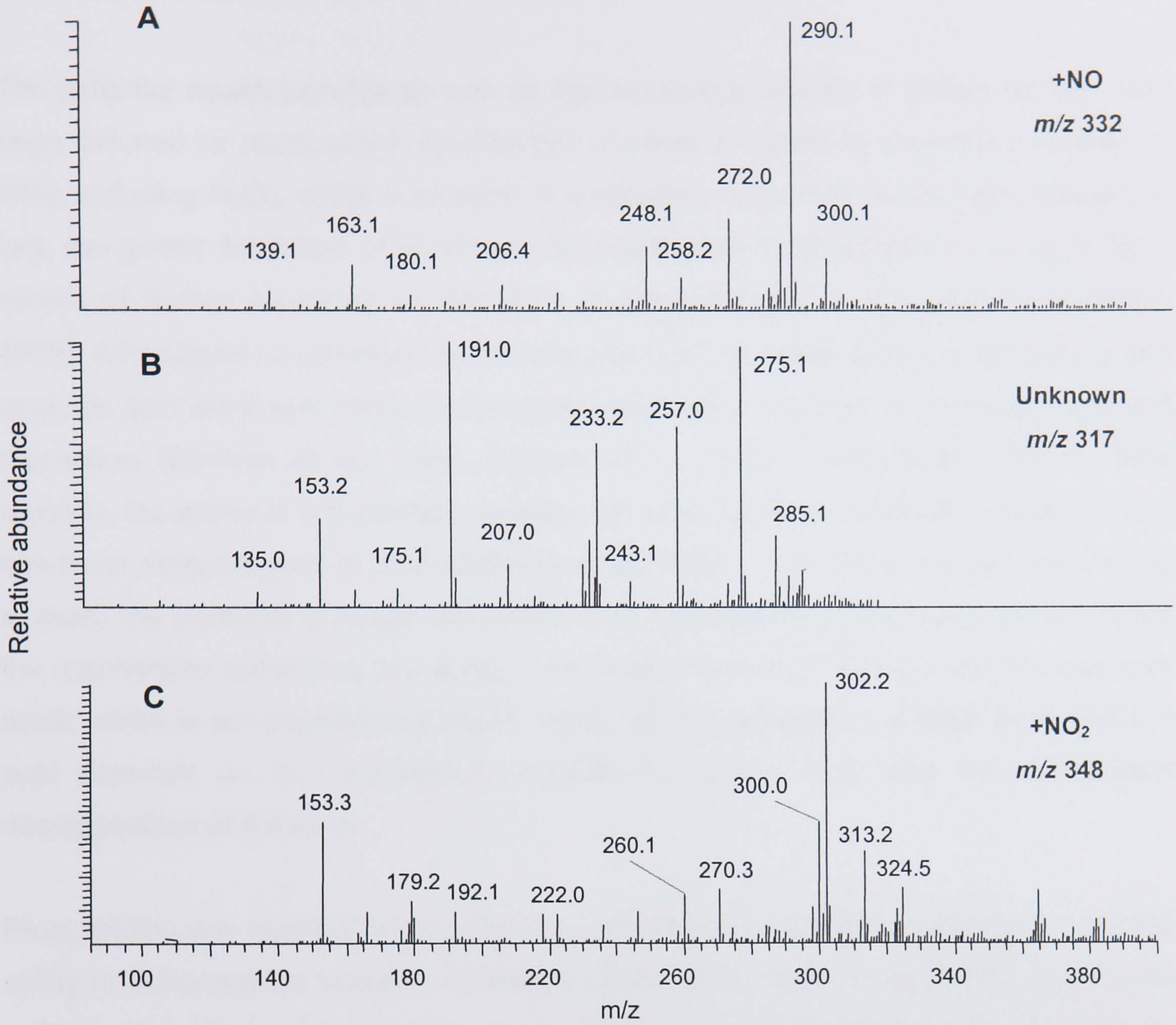


Fig 3.17 Product ion spectra for products of the reaction mixture from interaction between hesperetin and nitrite in acid. All spectra were recorded in the positive ion mode. Product ion spectra of (A) nitroso-hesperetin; (B) unknown product and (C) nitro-hesperetin. (This figure shows the m/z range from 100-400).

3.3 DISCUSSION

The potential health benefits as well as the deleterious effects of dietary nitrites have been debated for many years. Acidification of nitrite is known to generate a number of RNS, including N_2O_3, which is capable of nitrosating secondary amines and amides. In fact, the gastric formation of *N*-nitroso compounds has been suggested to be a major source of human exposure to this class of environmental carcinogens (Kyrtopoulos 1989). It has been established that compounds such as polyphenols, α-tocopherol and ascorbic acid react with nitrite preferentially, and hence are able to inhibit endogenous nitrosation (Bartsch *et al.,* 1986, Bartsch *et al.,* 1993, Tannenbaum 1989). More recently, the ability of polyphenols to inhibit tyrosine nitration mediated by nitrite in acid has been demonstrated *in vitro* (Oldreive *et al.,* 1998). This has prompted our interest to study the potential of cocoa procyanidins as scavengers of RNS, and to investigate the mechanism underlying this ability. The reaction between amines and phenols with acidic nitrite is pH-dependent (Challis 1970), as the generation of RNS from nitrite in acid depends on the protonation of nitrite to nitrous acid, and the subsequent decomposition of the latter.

Procyanidins are reported to be effective antioxidants and RNS scavengers, with the ability to scavenge peroxynitrite (Arteel and Sies 1999, Arteel *et al.,* 2000), superoxide radicals (Saint-Cricq De Gaulejac *et al.,* 1999) and nitric oxide radicals (Virgili *et al.,* 1998). Recently, the ability of catechin and their gallate esters (Pannala *et al.,* 1997) as well as epicatechin oligomers (Arteel and Sies 1999) to protect against peroxynitrite-mediated tyrosine nitration has been illustrated. Procyanidins can be obtained in our diet mainly from tea, red wine, apple and chocolate (Hammerstone *et al.,* 2000, Santos-Buelga and Scalbert 2000). Although chocolate may seem to be an unexpected source of dietary procyanidins, it can provide approximately 164.7 mg procyanidins per serving comparing to 147.1 mg from apples. Dark chocolate contains the largest amount of procyanidins by weight (4.45 mg/g), of which 1.08 mg/g is attributed to the monomers (Hammerstone *et al.,* 2000).

The present study demonstrates that procyanidins are good inhibitors of acidic nitrite-mediated tyrosine nitration under conditions akin to those in the gastric milieu. The IC_{50} of catechin monomer demonstrated in this study was 51.9 μM, in the presence of 400

μM nitrite and tyrosine. Although a dose-dependent inhibition of epicatechin dimer was not investigated, 66.9% inhibition was observed at 50 μM epicatechin dimer with 400 μM nitrite and 400 μM tyrosine. At equimolar concentration of procyanidins and nitrite, total inhibition of tyrosine nitration was achieved. It has previously been shown that the high nitrite concentration (400 μM) chosen in this study can be achieved in human gastric juice after the consumption of 100g of spinach (which can contain up to 500 mg of nitrate) (van Maanen *et al.,* 1998). Total procyanidins content (including monomer through decamer) present in a typical serving of dark chocolate (a 36.9 g bar of Galaxy, M&M Mars) was determined to be approximately 175 mg by Hammerstone *et al.*, (Hammerstone *et al.,* 2000). Assuming all the ingested procyanidins was in the form of monomer (owing to lack of information on the percentage composition of each oligomeric class in the chocolate sample), the concentration of procyanidins could reach up to 600 μM in the stomach after consumption of a 36.9 g bar of dark chocolate.

Inhibition of acidic nitrite-mediated tyrosine nitration by procyanidins was accompanied by the formation of new products, indicating that procyanidins competitively protect tyrosine from nitration induced by acidic nitrite-generated RNS due to a more rapid relative rate of reaction with the polyphenolic structures. When the products derived from the direct interaction of catechin monomer and epicatechin dimer with nitrite were compared to those obtained in the inhibition studies, similar retention times and UV spectra were obtained. These observations strongly support the suggestion that procyanidins protect tyrosine against nitration by reacting with the RNS derived from acidic nitrite in a sacrificial manner. In order to determine whether this reaction is likely to occur in a more physiologically relevant media, the same interaction was carried out in simulated gastric juice (pH of 1.5). The fact that the same products were produced in simulated gastric juice indicates the protective effects of procyanidins on acidic nitrite-mediated tyrosine nitration are likely to occur *in vivo*.

Nitration of monophenolates is a well-established mechanism on interaction with peroxynitrite (Kerry and Rice-Evans 1998, Pannala *et al.,* 1998). In contrast, it was found that the mechanism of interaction for catecholates upon exposure to peroxynitrite is electron donation, resulting in *o*-quinones formation (Kerry and Rice-Evans 1998, Pannala *et al.,* 1998). Based on this observations, it is anticipated that the main products formed between the direct interaction of procyanidins and nitrite in acidic

Interaction of flavonoids with acidic nitrite-generated reactive nitrogen species

medium would be either oxidation or nitration products. Furthermore, the study by Oldreive *et al.*, (Oldreive *et al.,* 1998) suggested that epicatechin inhibits tyrosine nitration mediated by acidic nitrite through a mechanism of oxidation. Contrary to the speculation that oxidation/nitration might be the predominant mechanism, mass spectrometric analysis has suggested an alternative mode of action for flavanols with acidic nitrite. For instance, the interaction of catechin monomer with acidic nitrite has revealed the formation of two products with identical *m/z* values of 347, an increase of 58 over the monomer (*m/z* for $[n\text{-}H^+]^+$ – 289). This led to the deduction that two nitroso groups were attached to some part of the phenolic ring system. Further fragmentation led to a loss in molecular weight of 36, possibly corresponding to two water molecules. As a result, dinitrosation was proposed to be the mechanism of action for catechin monomer against acidic nitrite-mediated interaction. The same reaction mechanism was observed for epicatechin monomer upon exposure to acidic nitrite, in which two dinitroso products were formed. Interestingly, the two products derived from catechin monomer had identical retention time and UV spectra as those derived from epicatechin monomer. Since catechin and epicatechin are epimers, it is possible that epimerisation occurred during incubation in acidic medium, giving rise to identical products.

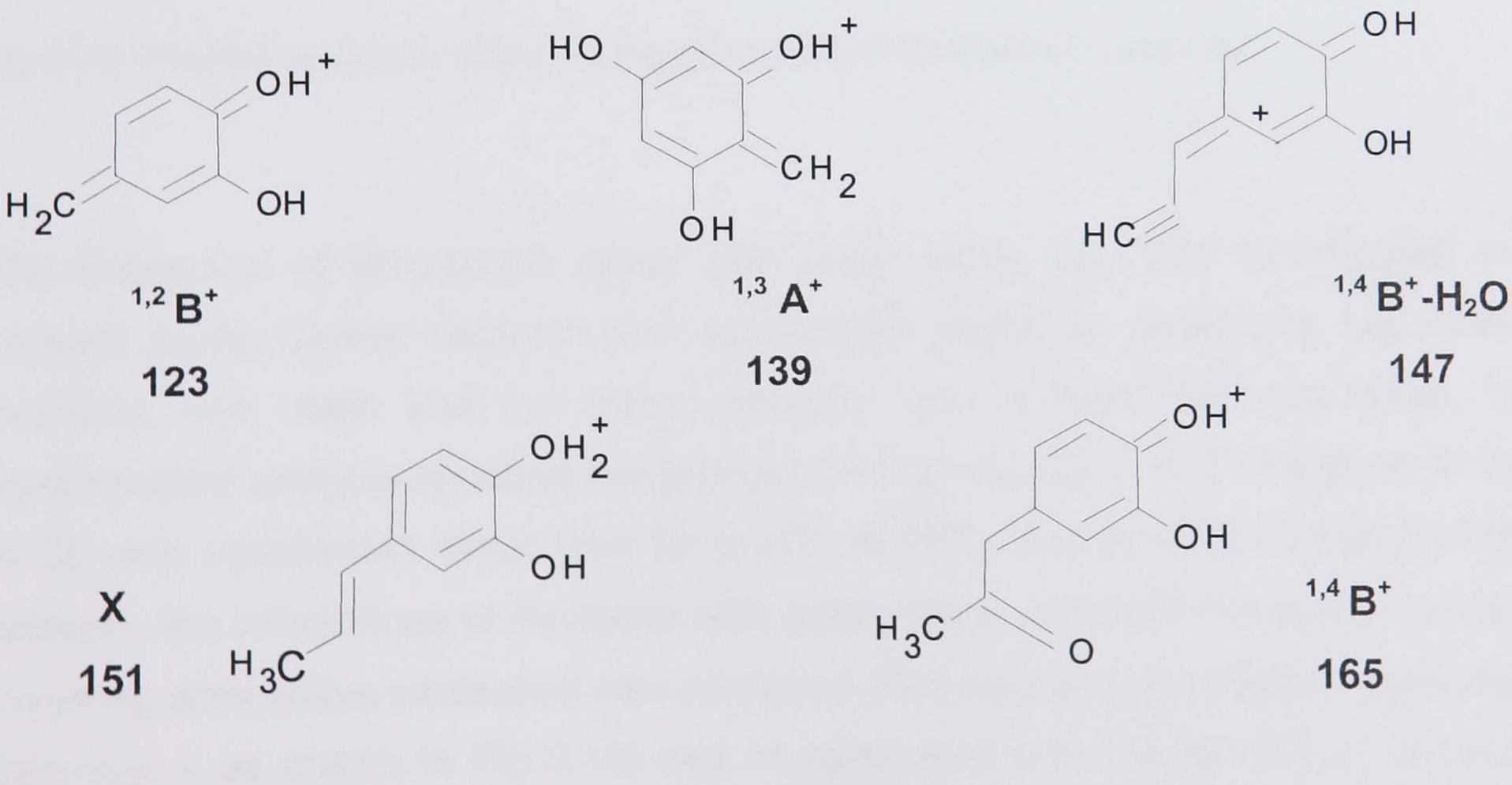


Fig 3.18 Nomenclature for fragments of epicatechin. Nomenclature of epicatechin MS/MS fragments as proposed by Ma *et al.*, (Ma 1997).

Interaction of flavonoids with acidic nitrite-generated reactive nitrogen species

The next question to be addressed was the exact location of nitrosation on procyanidins, which was unfortunately not possible to deduce accurately from the existing LC-MS/MS data. The product ion spectra of epicatechin and its reaction products with acidic nitrite were obtained using LC-MS/MS. All products showed a strong signal for the $^{1,2}B^+$ ion with *m/z* 123 for $[n\text{-}H^+]^+$, indicating the presence of a non-modified B ring (Fig 3.18). Furthermore, while a signal for the $^{1,3}A^+$ ion (*m/z* 139 for $[n\text{-}H^+]^+$) representing a non-modified A ring was detected in the unmodified epicatechin, it was not present in the ion spectra of the two nitroso products (Fig 3.13). As such it was concluded that nitrosation most probably took place on the A ring of catechin and epicatechin monomer upon exposure to acidic nitrite (Fig 3.19). However, further analysis such as NMR is required to confirm this speculation.

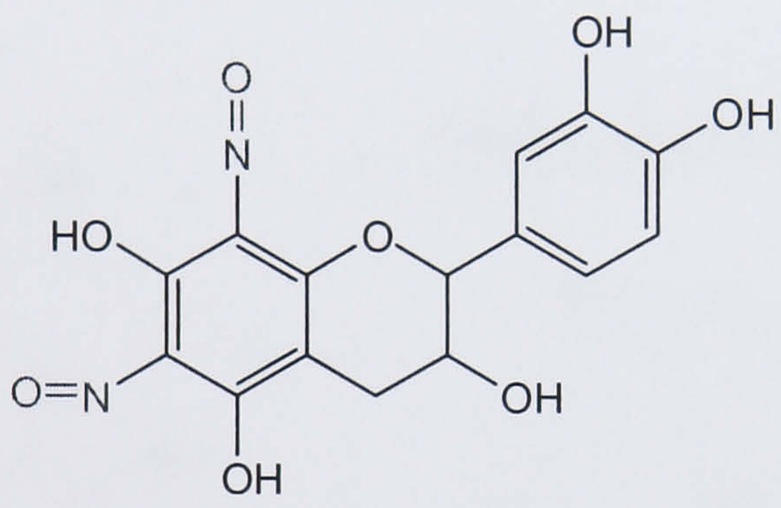
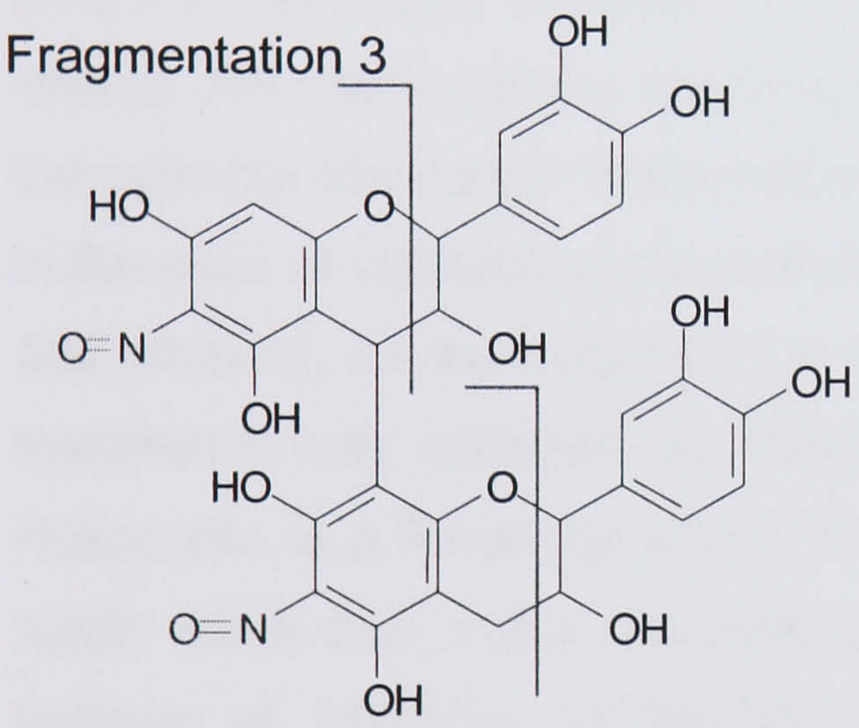


Fig 3.19 Possible structure of the dinitrosation product of catechin monomer.

The interaction of epicatechin dimer with acidic nitrite was also investigated in the present study. Unlike catechin and epicatechin monomer producing two dinitroso products, one major and two minor products were formed from the dimer. Mass spectrometric analysis revealed that product I has an *m/z* for $[n\text{-}H^+]^+$ of 635, an increase of 58 over epicatechin dimer (*m/z* for $[n\text{-}H^+]^+$ of 577). Based on the products formed between the interactions of monomer with acidic nitrite, a similar mechanism of action, involving competitive nitrosation was proposed. The fragmentation pattern (cleavage of fragment X as shown in Fig 3.18) was in agreement with the MS/MS of epicatechin dimer B2 in literature, resulting in cleavage of 152, which corresponds to the B ring structures (Holt *et al.*, 2002). As a result we proposed that nitrosation occurred on the A ring of the flavan-3-ol units upon exposure to acidic nitrite (Fig 3.20).

Interaction of flavonoids with acidic nitrite-generated reactive nitrogen species

Mass spectrometric analysis of product 2 of epicatechin dimer revealed a structure with molecular weight of 576. This was thought to be an anthocyanidin due to two reasons. The spectrum of product 2 is that typical of anthocyanidin, and secondly the depolymerisation of procyanidins and the subsequent release of anthocyanidins upon heating in acidic medium is well documented (Bate-Smith 1954, Porter 1986). Analysis of the last product revealed a structure with molecular weight of 607, thus leading to the speculation that it could be a mono-nitrosation product of the dimer. The fragmentation pattern of this product is similar to that of product I (Fig 3.20). As such, products 1 and 3 of dimer were suggested to be similar in identity, with the former being a dinitroso product and the latter a mono-nitroso product.



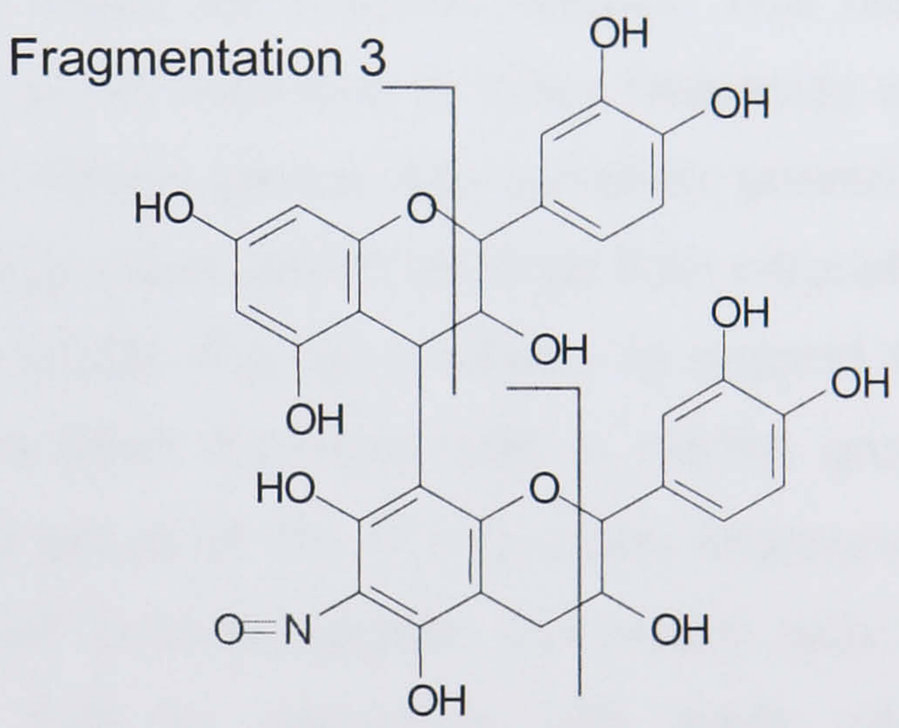


Fig 3.20 Possible structures of the dinitrosation and mono-nitrosation products of epicatechin dimer and their fragmentation pattern.

As mentioned before it has been suggested in the literature that polyphenols protect tyrosine against nitration by RNS via a mechanism of nitration or oxidation, depending on the presence of a catechol structure on the B ring (Kerry and Rice-Evans 1998, Oldreive, 1998 #306, Pannala *et al.,* 1998). However, the work described here

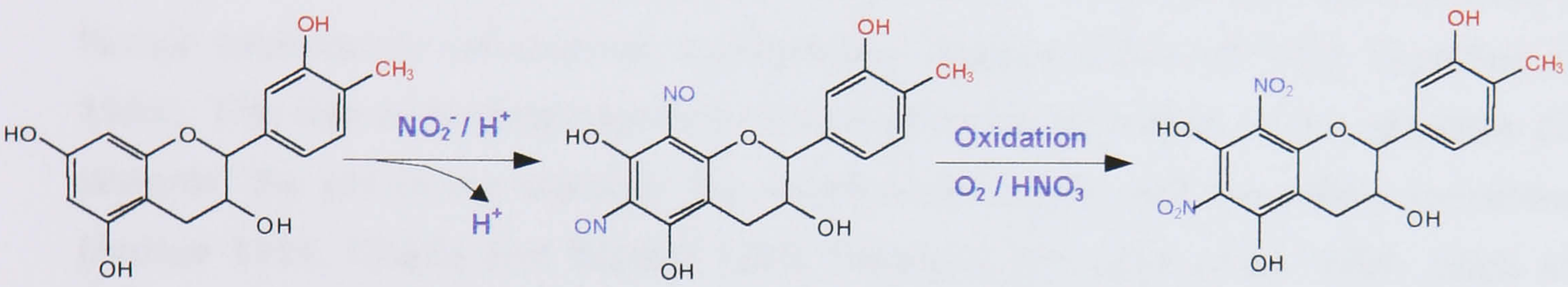
demonstrated that procyanidins inhibit acidic nitrite mediated-tyrosine nitration by undergoing competitive nitrosation. Formation of 3-nitrotyrosine from RNS generated by nitrite in acid has been proposed to proceed via a two-step mechanism (Section 1.3.3), involving an initial nitrosation step and the subsequent oxidation of the nitroso group to yield 3-nitrotyrosine (Knowles *et al.*, 1974). In contrast, the reactions between procyanidins and acidic nitrite involve only the nitrosation step with the reaction being terminated before the oxidation step. It is possible that the presence of the highly reducing catechol structure of flavanols prevents further oxidation of the nitroso groups. In order to understand the role of the catechol structure during interaction of flavanols with acidic nitrite, the interaction of Me-EC with acidic nitrite was investigated.

Interaction of Me-ECs resulted in formation of four products, with three of them being confirmed as the dinitroso-products of Me-ECs. In addition, small quantities of nitration products were also detected by LC-MS/MS within the reaction mixture. This result implies that the formation of nitroso products is not restricted to those flavonoids with the catechol structures. While oxidation of the nitroso groups was somehow prevented in the case of catechin and epicatechin, Me-ECs were able to undergo both nitrosation and nitration, as illustrated by LC-MS/MS analysis. Further evidence to support this hypothesis was obtained by investigating another flavonoid with a methyl group. Hesperetin is a flavanone with a 3′-O methyl group on the B ring. Upon exposure to acidic nitrite four major products were formed from hesperetin. Consistent with the findings of Me-ECs, LC-MS/MS revealed that on interaction with acidic nitrite hesperetin undergo both nitrosation and nitration. This strengthens the hypothesis that polyphenols without the catechol moiety can indeed be nitrated (as well as nitrosated) by acidic nitrite, whereas those with the catechol moiety can only be nitrosated, possibly due to their reducing abilities.

Taken together, it was hypothesised that nitration of polyphenols may proceed via a two-step mechanism similar to tyrosine nitration (Fig 3.21A). In the case of catechin and procyanidins, the presence of the catechol structures, possibly due to their reducing abilities, prevents the reaction proceeding to the subsequent oxidation step (Fig 3.21B). However, the exact mechanism of interaction i.e. which RNS generated from nitrite in acid is responsible/capable of carrying out the nitrosation is still not clear. It is known that under acidic conditions, the nitrosating agent N_2O_3 is formed from two

molecules of nitrous acid, which can react with unprotonated amine to form nitrosamines (Bartsch 1993). Certain amides can react with nitrosonium ion (NO^+) at pH <2, leading to the production of nitrosamide (Bartsch 1993). Further studies are required to determine whether or not these species are capable of nitrosating tyrosine under similar conditions, and thus aid our understanding of the mechanism involved in 3-nitrotyrosine formation by acidic nitrite.

A. Flavonoids without the catechol moiety, e.g. 3′ O-methyl epicatechin



B. Flavonoids with the catechol moiety, e.g. epicatechin

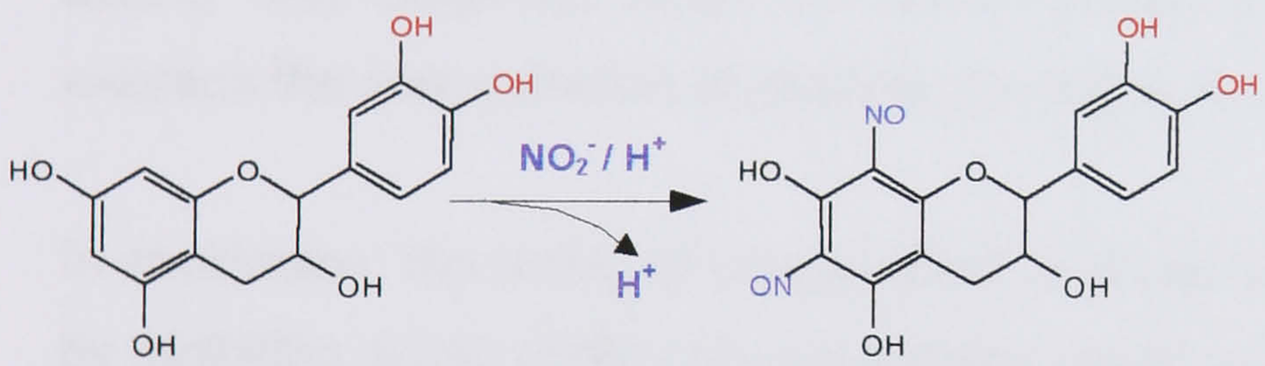


Fig 3.21 Proposed mechanisms of interaction between flavonoids and acidic nitrite. (A) Flavonoids without the catechol moiety are nitrated by acidic nitrite via a two-step mechanism; whereas (B) flavonoids with the catechol moiety are nitrosated.

In a recent publication Halliwell *et al.*, (Halliwell *et al.,* 2001) proposed that the mechanism of RNS scavenging by phenolic compounds may involve nitrosation. Indeed the nitrosation of phenolic compounds has been a subject of research for many years (Davies *et al.,* 1978, Knowles *et al.,* 1974, Knowles *et al.,* 1974, Pignatelli *et al.,* 1982, Rosenkranz *et al.,* 1990). It is known that nitrosation of phenolic compounds proceeds more rapidly than that of most secondary amines. In addition, it can happen

rapidly in a pH-independent manner from pH 1 to 5 (Challis 1973). Much attention has been focused on the ability of the resulting nitrosophenols to inhibit or catalyse N-nitrosation (Davies and McWeeny 1977). For instance, green tea extracts are reported to be capable of inhibiting the *in vitro* nitrosation of secondary amines, an effect mostly attributed to the catechins present in these extracts (Tanaka *et al.,* 1998). Similarly, caffeic acid, ferulic acid as well as tea and coffee that are rich in polyphenols inhibited endogenous formation of nitroso compounds in human subjects (Stich *et al.,* 1984, Wu *et al.,* 1993). On the other hand, readily oxidised phenolic compounds, particularly those containing the catechol moiety, were shown to catalyse the formation of N-nitrosamine from nitrite salts and secondary amines at gastric pH, and thus increase human exposure to carcinogenic substances (Challis and Bartlett 1975, Pignatelli *et al.,* 1982). The role of nitrosophenols in N-nitrosation is dependent on the structure of the phenols, the pH of the medium, the reaction conditions and the nitrite concentration (Archer 1984, Challis and Bartlett 1975, Gonzalez-Mancebo *et al.,* 1999, Stich *et al.,* 1984). The discrepancy of these results may lie in the fact that different experimental parameters were used in the investigations. Nevertheless, it was concluded that many naturally occurring polyphenols inhibit N-nitroso compound formation, with catalytic activity only observed when the concentration of the nitrosating agent significantly exceeds the concentration of phenols (Gonzalez-Mancebo *et al.,* 1999).

In conclusion, the ability of procyanidins to scavenge RNS can provide health benefits by inhibiting acidic nitrite-induced tyrosine nitration and N-nitrosamine formation in the gastric milieu. HPLC and LC-MS/MS analysis revealed the mechanism of protection by procyanidins is via competitive nitrosation. Future studies of the biological properties of nitrosated procyanidins are required to further understand the implications of dietary procyanidins on human health.

CHAPTER FOUR

Synthesis and purification of nitrosated catechins

4.1 OBJECTIVES

Thin layer chromatography (TLC) is routinely used in the fractionation of mixtures of compounds for both analytical and preparative studies. Thin layer chromatography, like all chromatographic techniques, involves a suitable adsorbent (the stationary phase), and solvents or solvent mixtures (the mobile phase) to separate and distribute the sample molecules. For TLC the adsorbent is coated as a thin layer onto a suitable support e.g. a glass plate, or an aluminium or polyester sheet. TLC is widely used for qualitative analysis of flavonoids because of the wide range of stationary phases available, such as alumina, silica, cellulose, polyamide, reverse phase silica, etc (Markham 1998). The adsorbent commonly used in TLC is silica, a stable material that is chemically resistant to most solvents. Silica is produced from orthosilicic acid, and because of the presence of silanol (Si-OH) groups it is very polar and hydrophilic. The particles are rigid, and unlike most resins, do not shrink or swell when exposed to solvents. Silica has been described as a good sorbent material because it was considered to be inert, and thus exhibit little non-selective adsorption of analyte. However, oxidation of nitrosated catechins was experienced in the present study, which would be described in detail in Section 4.2.2. The stability of silica is confined to the pH range 3-8.

About 80% of all TLC separations are performed with silica 60 i.e. with mean pore diameter 60 Å (6 nm). Pore size is inversely proportional to surface area, with smaller pore sizes offering better retention of analyte. Other factors affecting separation includes particle size and the thickness of the adsorbent layer. The particle size for a standard silica plate is between 5 and 17 μm, whereas the thickness of the layer is 0.25 mm for analytical plates and 1 mm for preparative plates.

The objective of this chapter was:

To develop a technique for the isolation and purification of NCs on a large scale.

The results and discussion will be combined in this chapter due to the nature of this work.

Synthesis and purification of nitrosated catechins

4.2 RESULTS AND DISCUSSION

4.2.1 Synthesis of nitrosated procyanidins

It was shown in chapter 3 that flavonoids have the ability to protect tyrosine against nitration mediated by acidic nitrite. Flavonoids containing a catechol moiety in the B ring undergo nitrosation on interaction with acidic nitrite. The work described in this thesis will be focusing on catechin and its nitrosated products, nitrosated catechin 1 (NC1) and nitrosated catechin 2 (NC2). To enable more comprehensive evaluations of the biological properties of NC1 and NC2, a method was developed to enable synthesis of NC1 and NC2 on a large scale using TLC and HPLC. Nitrosated catechin 1 and NC2 were synthesised by incubating 20 mM catechin and 20 mM nitrite in acid for 2h at 37°C.

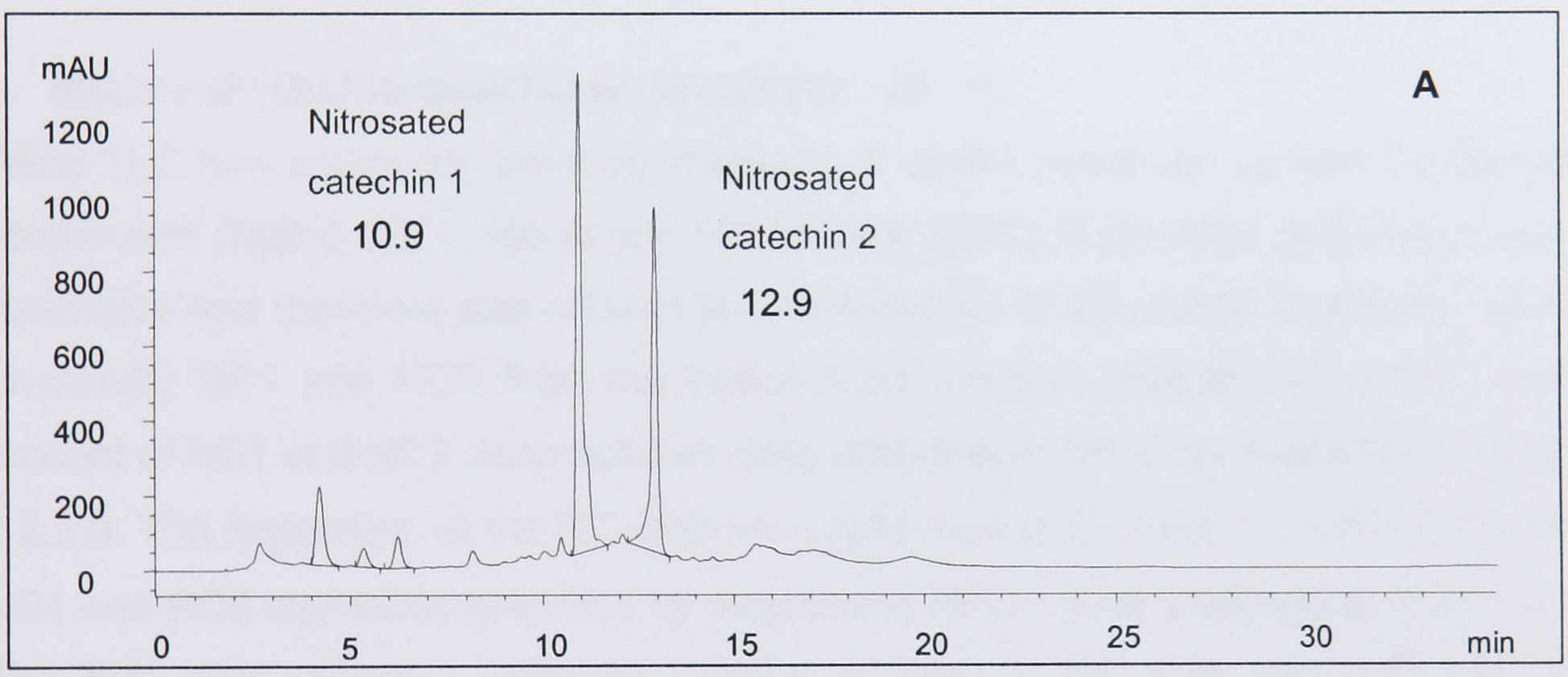


Fig. 4.1 Reverse phase HPLC analysis of the interaction between 20 mM catechin with 20 mM acidic nitrite in 0.5M HCl (A), the UV spectra for NC1 (B) and NC2 (C).

Synthesis and purification of nitrosated catechins

Fig 4.1A depicts the HPLC trace obtained following the interaction between 20 mM catechin and 20 mM nitrite in 0.5M HCl. The major peaks in the chromatogram, NC1 and NC2 had RT of 10.9 and 12.9 min, respectively. The spectra of NC1 and NC2 are illustrated in Fig 4.1B&C. The reaction mixture was freeze-dried and the resulting powder, approximately 1.5 g of nitrosated catechin preparation (NC prep) was subjected to purification using TLC and preparative HPLC.

4.2.2 Detection of nitrosated catechins by TLC

Isolation and purification of compounds using flash chromatography and preparative TLC required a suitable TLC method for fast and simple detection of NC1 and NC2. This section describes the development of a suitable TLC mobile system and detection method for the separation of NC1 and NC2 from NC prep.

1. Methanol : Dichloromethane : Water (10 : 10 : 1)

Silica TLC has previously been described as a useful screening system for flavonoid compounds (Mabry 1970, Males and Medic-Saric 2001). It provides good bonding with flavonoids and therefore was chosen for the detection of nitrosated catechins. In order to identify NC1 and NC2 from the freeze-dried reaction mixture (NC prep) a small amount of NC1 and NC2 were isolated using preparative HPLC as described in Section 2.2.2.5. The separation of the NC prep was performed at 2, 5 and 10 μl NC prep, while NC1 and NC2 standards prepared by preparative HPLC were analysed at 5 and 10 μl. The TLC plate obtained using the various volumes of NC prep and NC1 and NC2 standards is shown in Fig. 4.2. When examined under UV at 254 nm, a faint spot was seen using 5 μl NC prep whereas two faint spots were detected using 10 μl NC prep. In contrast, no spots were observed in either NC standard at 5 or 10 μl.

The plate was therefore sprayed with Naturstoff reagent, followed by 5% polyethylene glycol 4000 in 96% ethanol to test for the presence of flavonoids. When examined under UV light at 365 nm, the presence of flavonoids is indicated by fluorescent zones. Fluorescent zones were detected close to the sample loading points, suggesting no elution/separation of compounds occurred. In addition, Dragendorff's reagent was used to detect the presence of nitrogen-containing compounds, but no spot was detected. This is probably because a relatively large amount of nitrogen-containing compound is

required for detection and the amount of NCs present was below the detection limit. Since this mobile system did not result in an effective elution of NCs, the polarity of the mobile phase was increased to aid elution of polar nitrosated compounds.

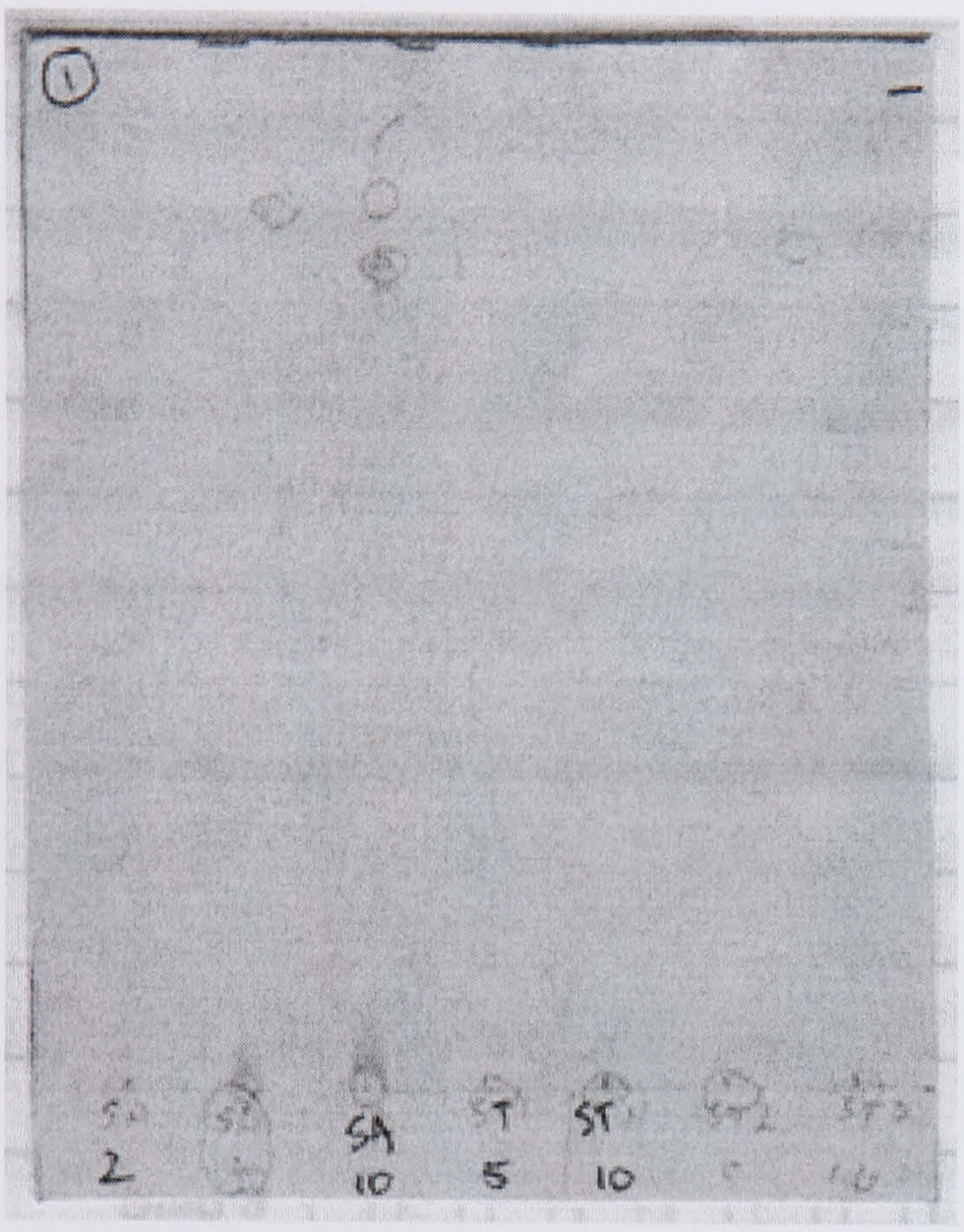


Fig 4.2 TLC analysis of NC prep, NC1 and NC2 using Methanol: Dichloromethane: Water (10: 10: 1).

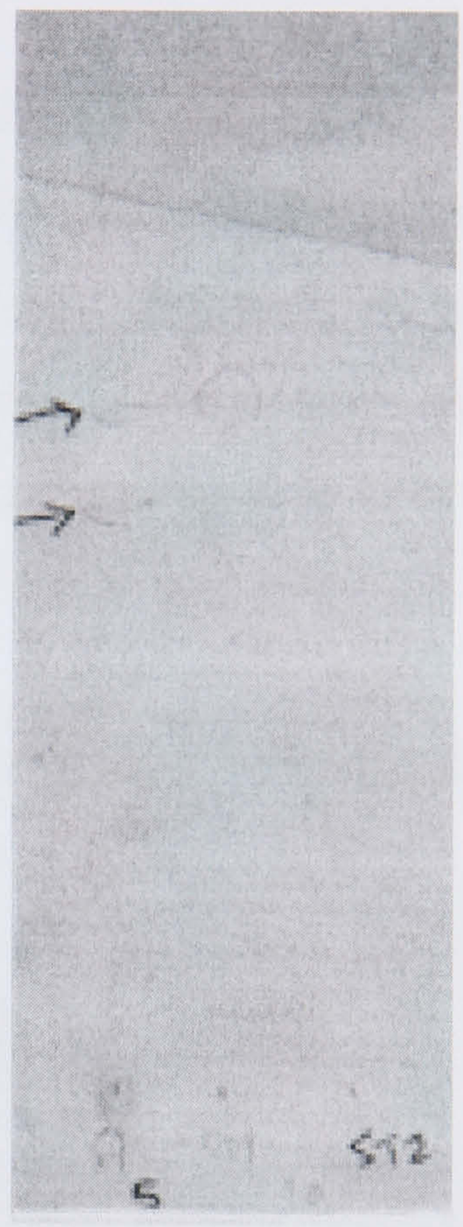


Fig 4.3 TLC analysis of NC prep, NC1 and NC2 using Methanol: Dichloromethane (80 : 20).

2. Methanol : Dichloromethane (80 : 20)

Using a mobile phase consisting predominantly of methanol for higher polarity, NC prep was analysed at 5 μl while NC1 and NC2 standards were analysed at 10 μl. As illustrated in Fig. 4.3, two spots were detected in NC prep when examined under UV at 254 nm. One spot was detected with the NC1 standard, but by contrast, nothing was detected with NC2 standard. The plate was sprayed with dragendoff's reagent but no nitrogen-containing compounds were detected.

Synthesis and purification of nitrosated catechins

Since the elution of NCs using methanol : dichloromethane (80 : 20) as mobile phase was not satisfactory, this system was concluded to be unsuitable for the detection of nitrosated catechins. An alternative mobile system was tested.

3. Ethyl acetate : Formic acid : Acetic acid : Water (100 : 11 : 11 : 27)

This mobile system has been used for the separation and detection of flavonoids in literature (Bloor 2001, Mabry 1970, Wagner 1983). NC prep was analysed at 10 μl, while NC1 and NC2 standards were analysed at 10 and 20 μl (Fig. 4.4). When examined under UV at 254 nm two spots were detected in NC prep while one spot was present in each of the NC1 and NC2 samples.

For qualitative evaluation of a TLC plate the retention factor (R_f value) of the spots can be calculated and compared. The R_f value is defined as follows:

$$R_f = \frac{\text{distance from start to centre of substance spot}}{\text{distance from start to solvent front}}$$

This is characteristic for any given compound using the same stationary phase and mobile phase for development of the plates. Identification of unknown spots can be achieved by comparing the R_f value to substances with known R_f values.

As shown in Fig 4.4 the R_f value (~ 0.74) of the lower spot in NC prep matches the R_f values of the spots in NC1 and NC2 standards, thus confirming the presence of NCs in the NC prep sample. The spot with higher R_f value (~ 0.81) in NC prep is likely to be catechin, as it is known that small amounts of unreacted catechin are present in the NC prep sample. On prolonged exposure to air a green colour started to develop in all NC spots. The intensity of the colour increased as the exposure time increased, suggesting oxidation of the samples. This is probably because silica is a strong oxidising agent owing to the presence of silanol groups (Si-OH). This mobile system was concluded to be satisfactory for the elution and detection of NCs, but not suitable for purification purposes as the compounds were prone to oxidation by the silica.

Synthesis and purification of nitrosated catechins

Analysis of flavonoids and nitrosated catechins is routinely carried out using a reverse phase HPLC system in our laboratory. The sorbent used in reverse phase TLC plates is C_{18} octadecyl with a long hydrocarbon chain, providing a non-polar surface (Fig 4.6). This has the advantage over TLC in that it is not oxidising, and consequently was tested for its suitability in the separation of NCs.

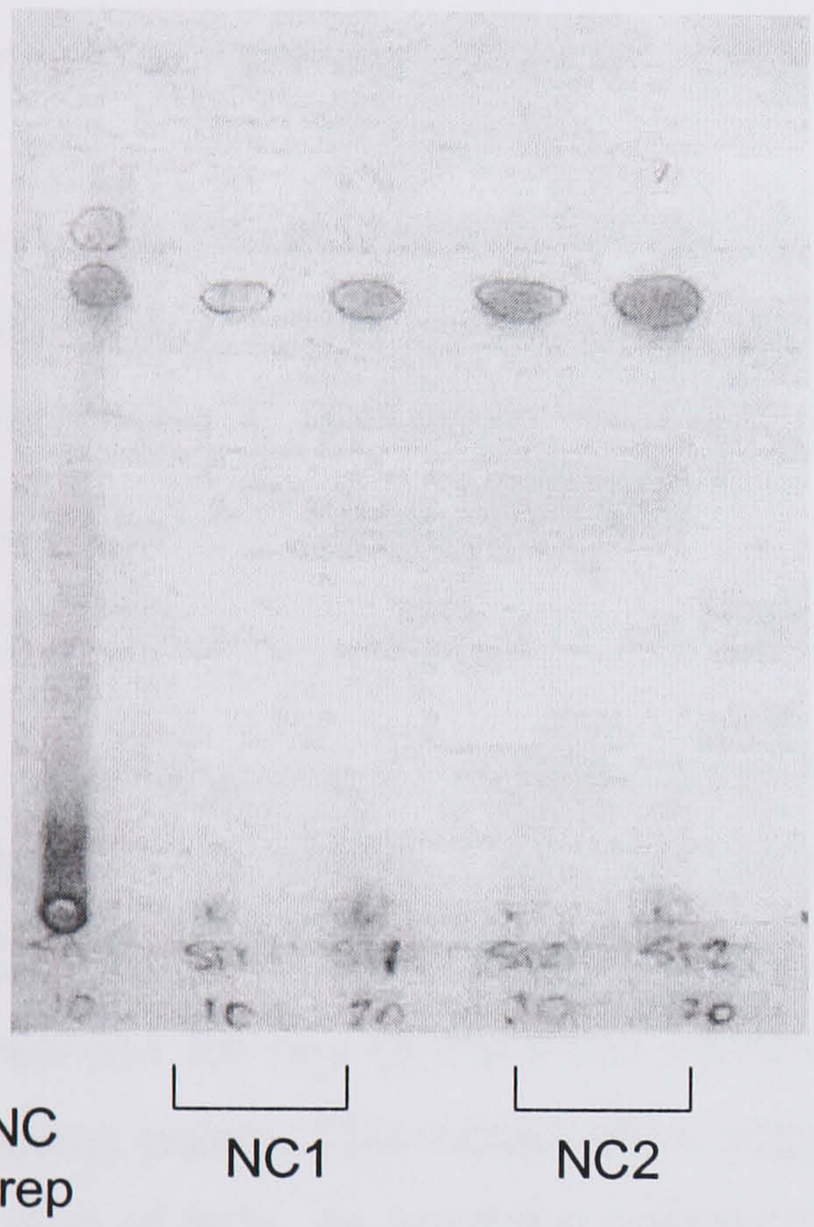


Fig 4.4 TLC analysis of NC prep, NC1 and NC2 using Ethyl acetate: Formic acid: Acetic acid: Water (110: 11: 11: 16)

Fig 4.5 TLC analysis of NC prep, NC1 and NC2 using MeOH: H_2O: HCl (50 : 50 : 0.1)

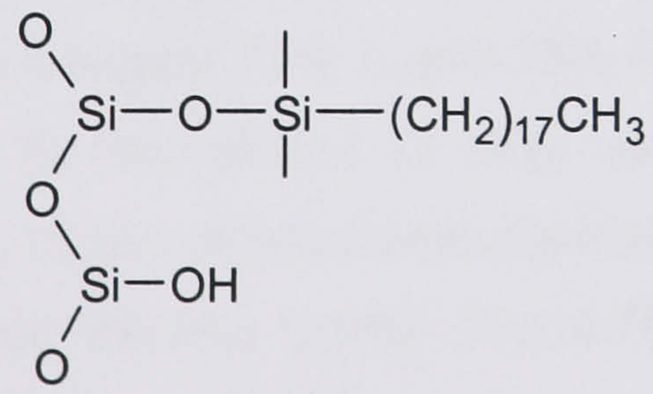


Fig 4.6 Chemical structure of C_{18} octadecyl.

Synthesis and purification of nitrosated catechins

4. MeOH : H_2O: HCl (50 : 50 : 0.1)

The first mobile phase tested on the reverse phase system was similar to the mobile phase A (5% MeOH, 0.1% HCl in H_2O) used in routine HPLC analysis, as described in Section 2.2.1.4. The polarity of this mobile system was reduced compared to mobile phase A in HPLC analysis by increasing the percentage of MeOH to aid separation of NCs (all compounds would resolve close to solvent front if mobile system is too polar). As shown in Fig 4.5, NC1 and NC2 remained at the point where they were loaded. By contrast no apparent spot was detected at the point of loading for the NC prep sample.

Since this mobile system did not result in a good separation of the NC prep sample, additional systems were tested, consisting of varying proportions of mobile phases A (5% MeOH, 0.1% HCl in H_2O) and B (acetonitrile) typical of those used in the HPLC analysis of nitrosated catechins.

5. Mobile phase A : mobile phase B (100 : 0)

100% mobile phase A was first tested for the elution of NCs from NC prep, NC1 and NC2 standards using reverse phase TLC (Fig. 4.7A). No movement of compound was observed for any of the three samples, as shown by the strong colour retention at the loading points. This observation suggests that the mobile system was too polar for the elution of NCs. As such the polarity of the mobile phase was reduced (see below).

6. Mobile phase A : mobile phase B (75 : 25)

As shown in Fig 4.1A, the elution times of NC1 and NC2 are approximately 10.9 and 12.9 min, respectively. The composition of the mobile phase at the time of nitrosated catechins elution was approximately 75% A and 25% B. As a result this mobile system was prepared and tested for the elution of NCs using reverse phase TLC. When sprayed with anisaldehyde, brown streaks were detected for the NC1 standard and NC prep samples close to their loading points (Fig 4.7B), suggesting the presence of flavonoids. For the NC2 standard, a yellow spot was observed at the point of loading, suggesting a less polar solvent might be required to assist elution of this compound. Although a spot was observed for the NC2 sample, which may be the NCs, no movement was seen in NC1.

Synthesis and purification of nitrosated catechins

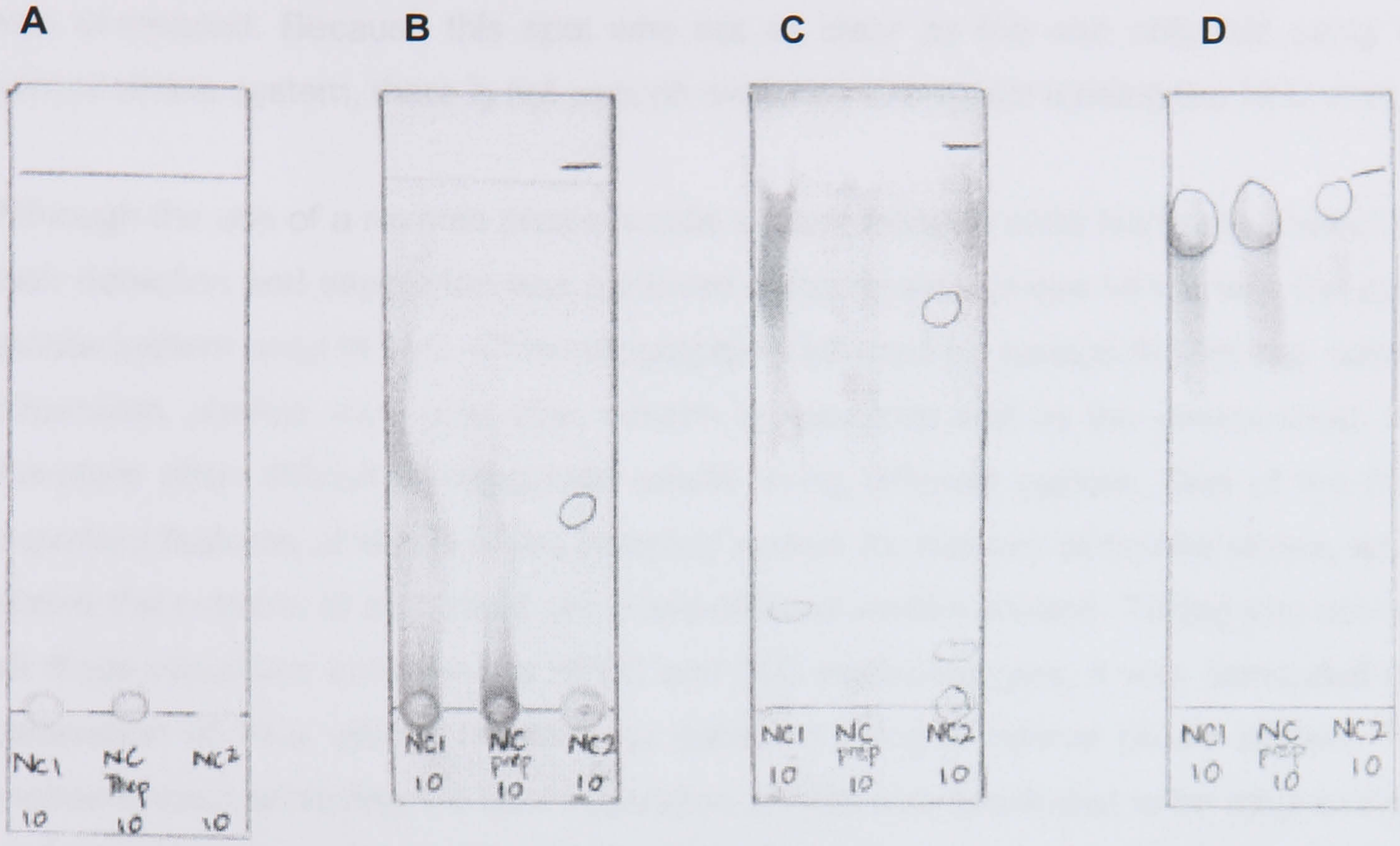


Fig 4.7 TLC analysis of NC prep, NC1 and NC2 using (A) 100% mobile phase A; (B) 75% mobile phase A; (C) 50 % mobile phase A and (D) 25% mobile phase A.

7. Mobile phase A : mobile phase B (50 : 50)

The proportion of mobile phase B was increased to 50% in order to facilitate elution of NCs. After spraying with anisaldehyde, the brown streaks in the NC1 standard moved closer to the solvent front. However, again no defined spot was detected to indicate separation of nitrosated catechins (Fig 4.7C). As for NC2, a faint yellow spot was detected with very low R_f value. The absence of a NCs spot in the NC prep sample was probably owing to interaction with impurities present in the NC prep sample. This could possibly influence the bonding of NCs with the C_{18} reverse phase material, thereby affecting the efficiency in separation.

8. Mobile phase A : mobile phase B (25 : 75)

When the polarity of the mobile system was decreased using 75% mobile phase B, the brown streaks were concentrated close to the solvent front (Fig 4.7D). However, separation of NCs and NC2 from NC prep was still not achieved. The yellow spot

detected in NC2 eluted closer to the solvent front as the polarity of the mobile phase was decreased. Because this spot was not as clear as the one obtained using the normal phase system, there is not enough evidence to suggest it being the NC2 spot.

Although the use of a reverse phase mobile system failed to elute Nitrosated catechins, their detection and separation was achieved using reverse phase HPLC with the same mobile system used in TLC. Chromatography is affected by various factors e.g. column dimension, particle size, pore size, column temperature well as the environment. It is therefore often difficult to reproduce results using different system. One of the most important features of HPLC is the pumping system for delivery of mobile phase, which allows the creation of a gradient using two different mobile phases. Taking into account all these variations between the HPLC and TLC methodologies, it was concluded that separation of NCs with TLC was best achieved using a normal phase system. The mobile system achieving the best separation of NCs was concluded to be ethyl acetate: formic acid: acetic acid: water (100: 11: 11: 27), and hence used for all remaining TLC analysis.

4.2.3 Flash chromatography

Flash chromatography is a rapid purification technique used for isolation/purification of compounds. As in standard liquid chromatography, the separation takes place in a column packed with stationary phase. The sample is applied to the head of the column with the mobile phase under positive pressure, usually applied using a nitrogen cylinder at about 10 bars. As such rapid separation of compounds can be achieved. Enhanced resolution can be achieved by the use of small particle, narrow size range adsorbents. To detect for the presence of the compound of interest, samples are usually taken from collected fractions and analysed using TLC.

1st analysis

Twenty seven fractions were initially collected using flash chromatography, and TLC analysis was carried out using ethyl acetate: formic acid: acetic acid: water (100: 11: 11: 27) as mobile phase. TLC analysis was carried out with every three fractions (5-20 μl) to roughly locate the presence of NCs.

Synthesis and purification of nitrosated catechins

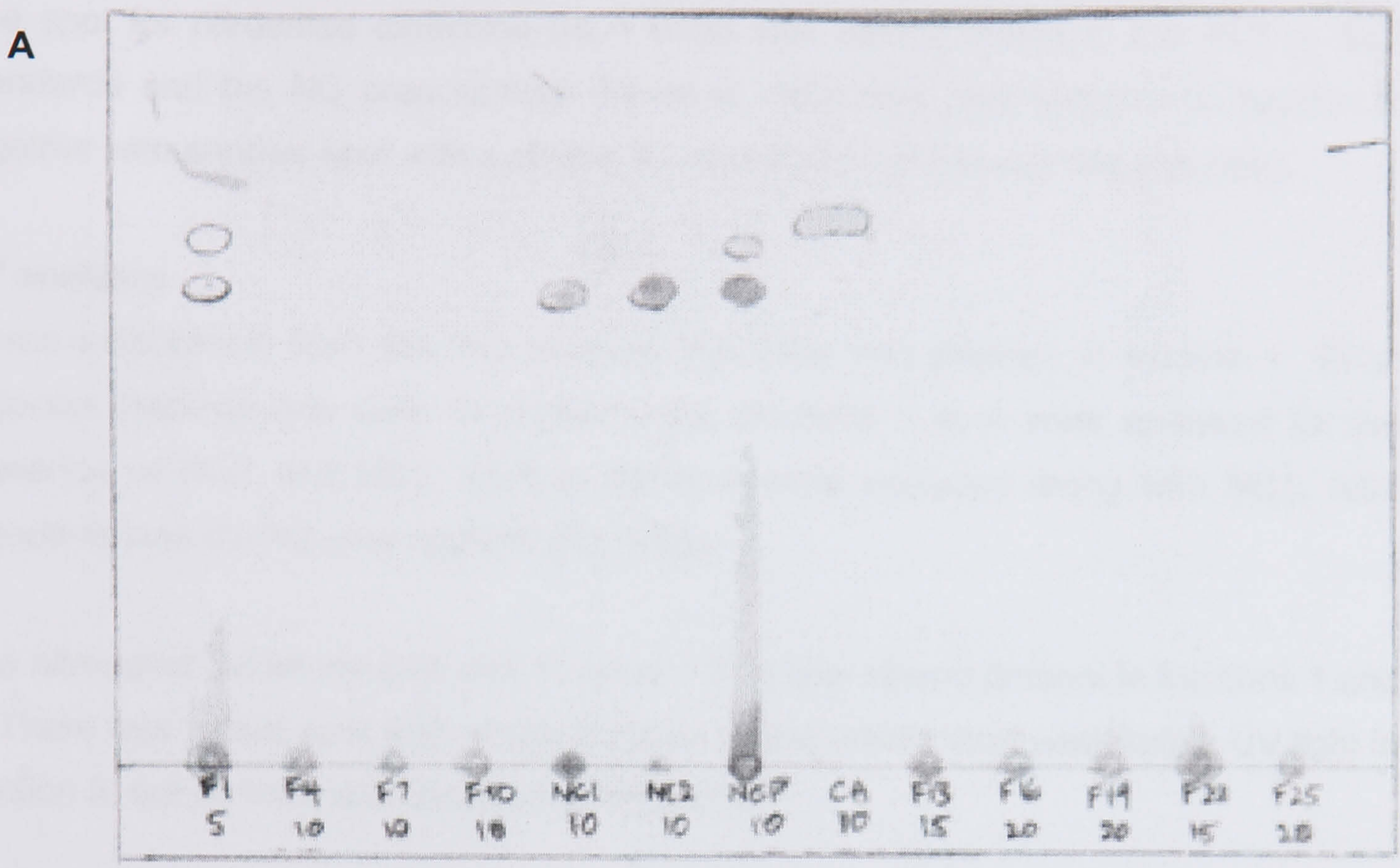


Fig. 4.8 TLC analysis of fractions obtained using flash chromatography. (A) 1st analysis; (B) 2nd analysis and (C) 3rd analysis.

Synthesis and purification of nitrosated catechins

Fig 4.8A depicts the TLC analysis of fractions obtained using flash chromatography. The spot for nitrosated catechins (R_f = 0.74) was clearly visible in the NC1 & NC2 standards and the NC prep sample. However, NCs was only detected in fraction 1, together with another spot with a similar R_f value (0.81) as the catechin standard.

2nd analysis

It was established from the first analysis that NCs was present in fraction 1. Since adjacent fractions are likely to contain NCs, fractions 1 to 4 were analysed for the presence of NC1 and NC2. All four fractions were analysed along with NC1, NC2 standards and the NC prep sample (Fig 4.8B).

The nitrosated catechins spot with R_f value ~ 0.74 was clearly present in fractions 1 and 2. There was a faint spot with similar R_f value visible under short wavelength UV light in fraction 3, but no NCs was detected in fraction 4.

3rd analysis

In the previous analysis only a 10 µl aliquot of fraction 4 was tested, from which no NCs was detected. The amount of NCs present in this fraction was possibly below the limit of detection. To ensure this was not the problem, fractions 4,5 and 6 were analysed at 30 µl (Fig 4.8C). Very faint spots with R_f value 0.74, similar to that seen with the NC1 standard, were detected in all samples under UV light (254 nm).

The fractions (1, 2 and 3) containing higher amounts of NCs were combined and freeze-dried, which was named, group 1. Fractions 4, 5 and 6 containing lower amounts of nitrosated catechins were also combined and freeze-dried (group 2). The weights of the samples were determined to be 175.6 mg and 24.5 mg for group 1 and 2, respectively. Further TLC analysis was carried out to confirm the presence of NCs in groups 1 and 2 before preparative TLC.

TLC of group 1 & 2

Group 1 and 2 were analysed using the same TLC system as described above. Two spots were detected in group 1, group 2 and NC prep samples when the plate was

examined under short range UV light (254 nm) (Fig 4.9A). The spot with lower R_f value (0.74) corresponds to NCs and the spot with higher R_f value (0.81) corresponds to catechin. The plate was also sprayed with DPPH$^{\bullet}$ radical, which is a widely used method to determine antioxidant activity of natural plant extracts (Brand-Williams 1995). DPPH$^{\bullet}$ is a stable radical with an absorption band at 515 nm. It loses the absorption when reduced by an antioxidant. When used as a spray agent on TLC, active compounds appear as yellow spots against a purple background. After spraying with DPPH$^{\bullet}$ radical, yellow colours were developed in the NCs and catechin spots isolated from group 1, group 2 and NC prep (Fig 4.9B). Interestingly, yellow streaks were also developed on the plate possibly due to nitrosated catechins and catechin being retained, and hence the strong antioxidant property. This was identified as a major problem during prep TLC, which would be described in detail in Section 4.2.4.1.

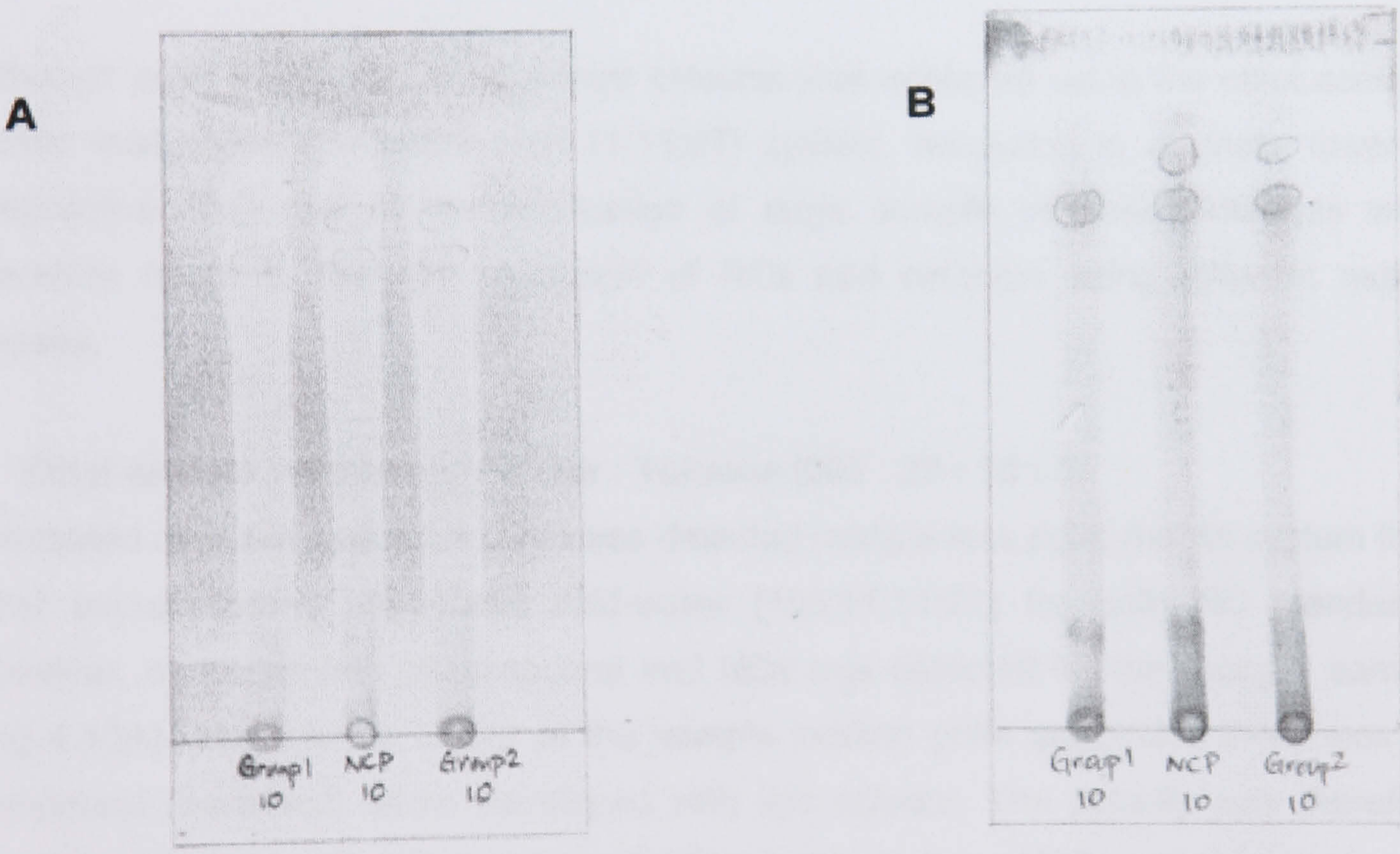


Fig 4.9 TLC analysis of fractions isolated using flash chromatography.

The same analysis was repeated for the identification of the impurities within group 1 and 2 (Fig 4.9B). The plate was sprayed with anisaldehyde, which detects a wide variety of natural compounds e.g., steroids, carbohydrates, phenols, glycosides, sapogenins, antioxidants etc. Upon heating a range of colours form specific for different

Synthesis and purification of nitrosated catechins

groups of compounds. When the plate was sprayed with anisaldehyde and heated, only yellow/brown streaks were detected, indicating the presence of flavonoids. No further attempt was made to characterise the impurities present in group 1 and 2.

4.2.4 Preparative TLC

Preparative TLC is useful for purification purpose since it is one of the simplest and cheapest methods available for the isolation of compounds. The plates used in preparative TLC are usually thicker than that used in analytical techniques. The techniques involved are similar to that described for analytical TLC previously. However, preparative TLC is labour intensive and is limited by the size of the sample that can be applied, which is usually less than 40 mg in total. Nevertheless it is still the most commonly used method in the final stages of compounds purification.

Although good separation of NCs from catechin was achieved using the ethyl acetate-formic acid-acetic acid-water (100:11:11:27) system, resolution is normally lower in preparative TLC due to the application of large sample volumes. Attempts were therefore made to test the resolution of NCs and catechin using different mobile phases.

1. Ethyl acetate : Methanol : Water : Toluene (200 : 27 : 10 : 5)

Nitrosated catechin spot (R_f = 0.75) was detected using a less polar mobile system than ethyl acetate-formic acid-acetic acid-water (100:11:11:27) for both NC standards. However, no separation of compound and NCs was detected for the group 1 sample (Fig 4.10A). The intense colour at the sample loading point suggested there was no compound movement when developed with this solvent. The polarity was therefore increased in the next TLC test to help facilitate better elution of NCs and catechin.

2. Ethyl acetate : Methanol : Water : Toluene (113.5 : 113.5 : 10 : 5)

In contrast to the original ethyl acetate-formic acid-acetic acid-water (100:11:11:27) system where good resolution of nitrosated catechins and catechin was achieved, separation of these two compounds was unsatisfactory when the polarity was increased by using equal volumes of ethyl acetate and methanol (Fig 4.10B). The R_f values for NCs and catechin were 0.72 and 0.73, respectively. No separation of NCs and catechin

from group 1 was achieved using this mobile system. The polarity of the developing solvent was therefore increased further to test for better separation of these two compounds.

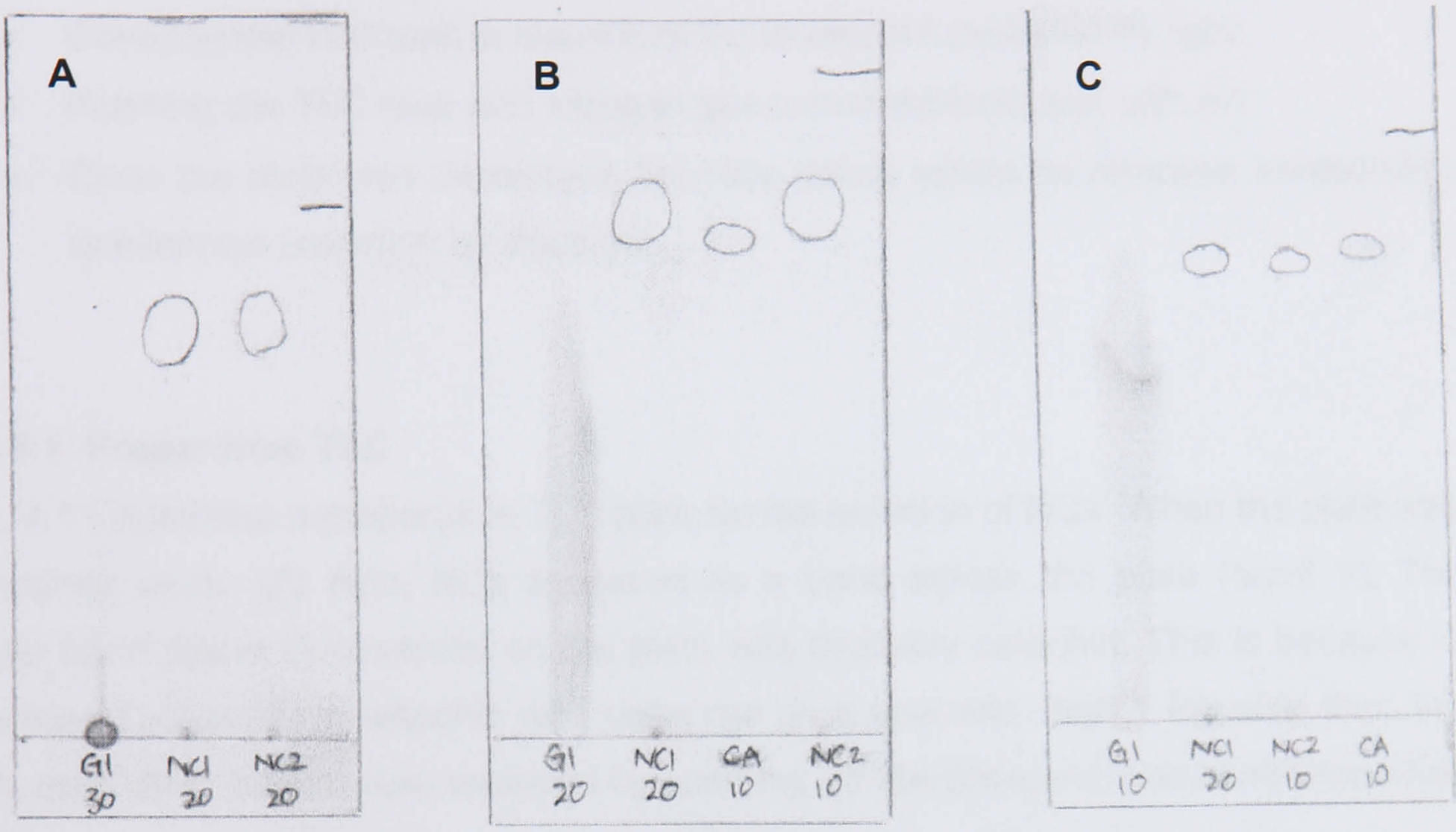


Fig 4.10 TLC analysis of different mobile phase for the separation of catechin and NCs in preparative TLC. (A) Ethyl acetate : Methanol : Water : Toluene (200 : 27 : 10 : 5); (B) Ethyl acetate : Methanol : Water : Toluene (113.5 : 113.5 : 10 : 5); Ethyl acetate : Methanol : Water : Toluene (27 : 200 : 10 : 5).

3. Ethyl acetate : Methanol : Water : Toluene (27 : 200 : 10 : 5)

Similar to the previous solvent system, large spots (R_f value = 0.81) were detected for both NC standards (Fig 4.10C). Increasing polarity of the mobile system did not facilitate separation of nitrosated catechins and catechin, as observed by the close elution of NCs and catechin, suggesting co-elution might occur using preparative TLC. The increase in mobile phase polarity resulted in a yellow streak developing for group 1, but no separation of NCs and catechin was observed. Furthermore, this mobile system did not enable separation of NCs from the mixture in group 1.

It was concluded that the best separation of NCs and catechin was achieved by using the ethyl acetate-formic acid-acetic acid-water (100:11:11:27) system, the mobile system of choice in preparative TLC. However, the drawback of this system is oxidation

Synthesis and purification of nitrosated catechins

of the nitrosated catechins, and special care has to be taken during development of the plates. Consequently, several precautions should therefore be taken to minimise the extent of oxidation:

- Covering the TLC tank in aluminium foil to prevent oxidation by light.
- Flushing the TLC tank with nitrogen gas to minimise contact with air.
- Once the plate was developed, the NCs bands would be removed immediately to minimise oxidation by silica gel.

4.2.4.1 Preparative TLC

Fig. 4.11 illustrates a preparative TLC plate for the isolation of NCs. When the plate was examined under UV light, NCs appeared as a band across the plate (band 1). The upper band (band 2) observed on the plate was probably catechin. This is because in previous TLC analysis catechin was observed as a spot with higher R_f value than the NCs spot. Both bands were removed by scarping off the silica and extracted from silica separately using methanol.

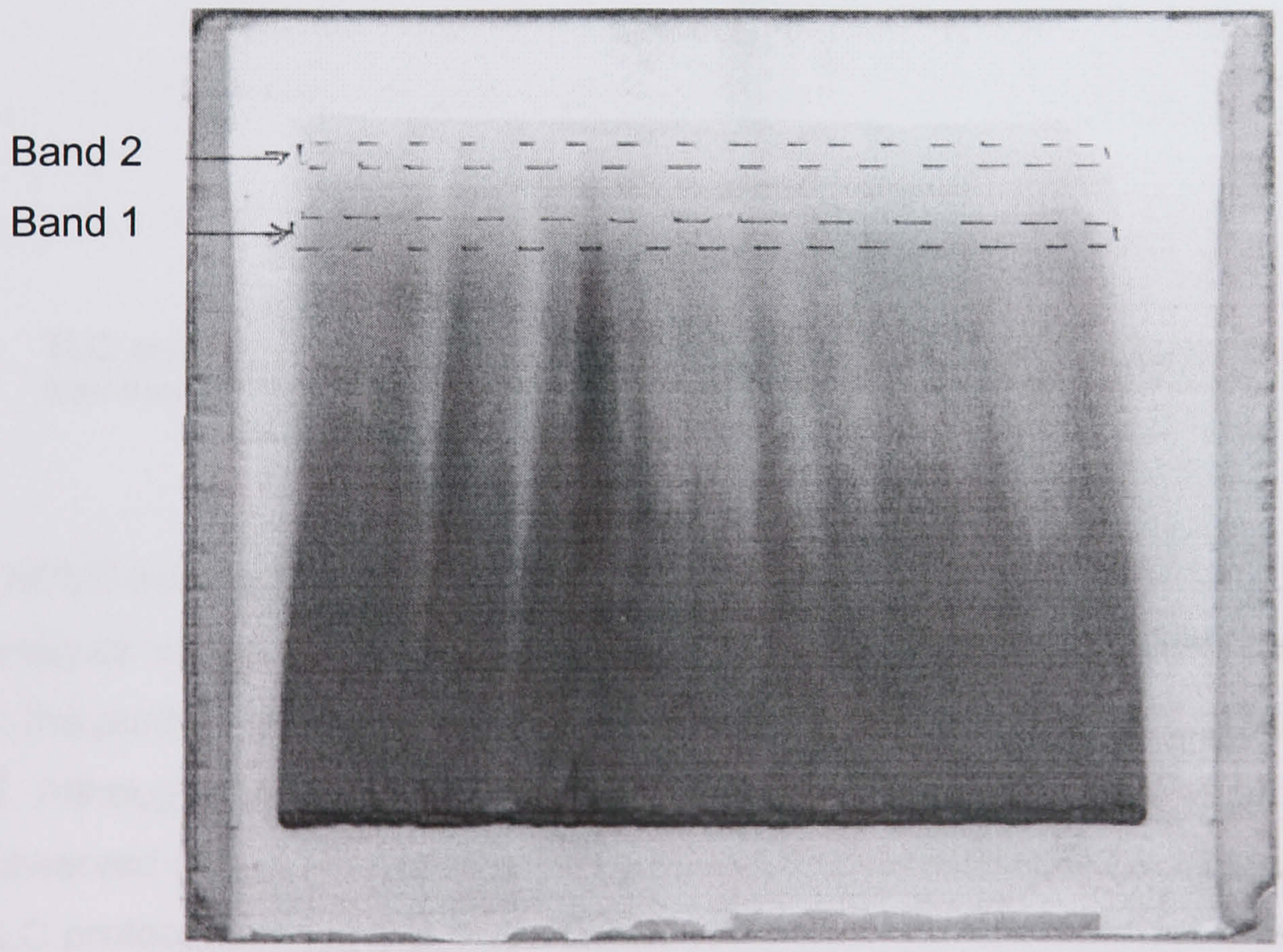


Fig 4.11 A representative preparative TLC plate used for the isolation of NCs using ethyl acetate-formic acid-acetic acid-water (100:11:11:27). The green colour resulted from the oxidation of the compounds, causing the streaky appearance of the plate.

Synthesis and purification of nitrosated catechins

After extraction of the compounds from bands 1 and 2 using methanol, they were subsequently analysed using TLC for the presence of nitrosated catechins. Band 1 was confirmed to be NCs, as it had a similar R_f value (0.74) as the NC standards. Although no catechin standard was used, band 2 had a higher R_f value than band 1 and was likely to be catechin. Isolation and purification of NCs using preparative TLC was concluded to be satisfactory, as the extract purity was much higher compared to group 3, a fraction obtained from flash chromatography.

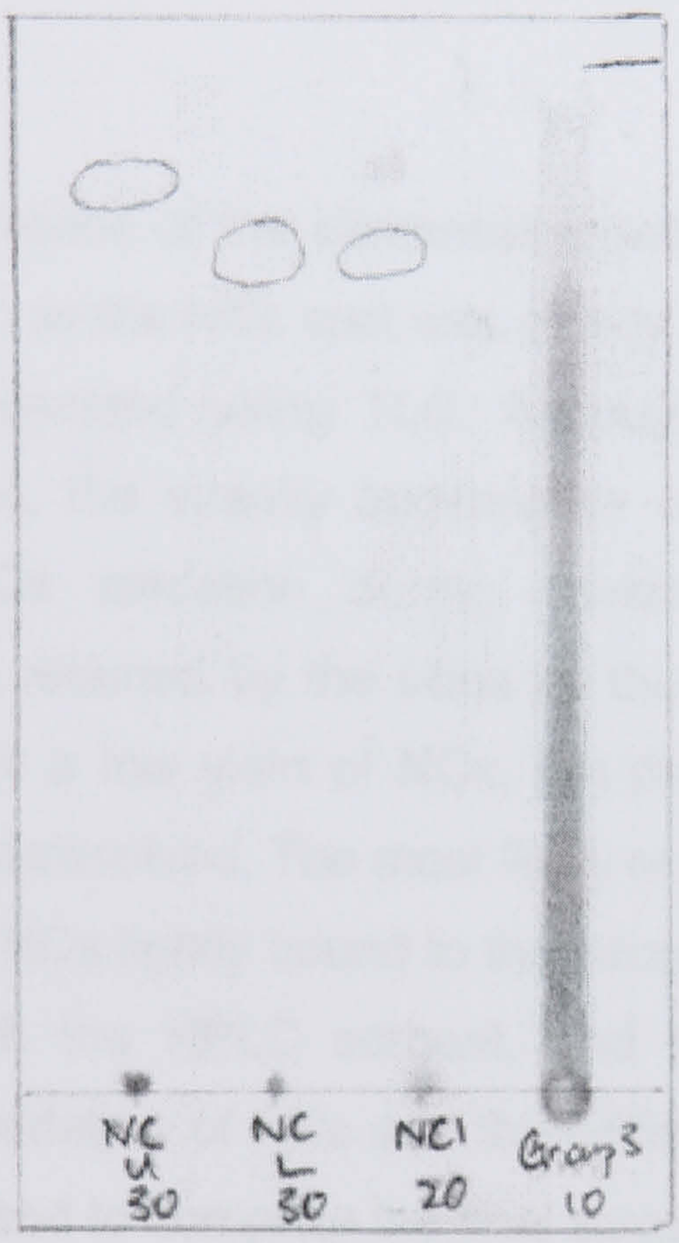


Fig 4.12 TLC analysis of the samples isolated by preparative TLC. NCU was band 2, which was thought to be catechin, and NCL was band 1, which was thought to be NCs.

4.2.4.2 HPLC analysis

HPLC analysis of the extracts was performed to help characterise band 1 and 2. In addition, the purity of the samples was examined to determine if further purification was required. Although the presence of nitrosated catechins in the extract of band 1 was clearly observed (Fig 4.12), no NCs was detected in the extracts using HPLC analysis. The HPLC protocol used in this analysis was identical to the one used for the detection of NCs in Section 4.2.1. When a standard of NCs (isolated using preparative HPLC) was analysed the nitrosated catechins peaks were present with similar retention time as

described in section 4.2.1. This ruled out the possibility that the absence of NC in the band 1 extract was caused by a problem with the HPLC protocol. It is not clear why NCs could not be detected by HPLC, but several suggestions have been proposed to account for the absence of NCs band:

- Inefficient extraction of NCs from silica with methanol.
- NCs was oxidised by silica during preparative TLC.
- NCs was tightly bound to silica post-extraction, thereby interfering with HPLC analysis.

It is unlikely that the absence of the nitrosated catechin peak resulted from inefficient extraction from the silica, as the NCs spot was clearly detected with a similar R_f value to the standards when separated using TLC. Although efforts were made to prevent oxidation of the samples, the streaky appearance of the preparative TLC plate (Fig 4.11) suggest that NCs oxidation during development of the plate occurred. Consequently, NCs was retained by the silica as the plate was developing. However, this can only account for a low yield of NCs, but the discrepancy between TLC and HPLC analysis was still unresolved. The most likely explanation was that extraction with methanol resulted in the NCs tightly bound to the silica. This would affect the bonding of nitrosated catechins with the HPLC sorbent, and thus its detection on the HPLC system. In view of the oxidation of NCs and the difficulty in their extraction from silica, preparative HPLC was used to complete the final stage of isolation.

The advantage of preparative HPLC over preparative TLC is that the former is essentially the same as analytical HPLC, but on a larger scale. The conditions applied in preparative HPLC, apart from the column diameter, flow rate and the particle size, can be adjusted to resemble those used in analytical HPLC. In the present study the sorbent material (C_{18} octadecyl), mobile phases (A – 10% methanol, 0.1% 5N HCl in water, and B – 50% acetonitrile, 0.1% 5N HCl in water) and the gradient were exactly the same as the analytical conditions used previously, which resulted in satisfactory separation of NC1 and NC2. Moreover, in preparative HPLC nitrosated catechins are not exposed to air during separation, thereby reducing their chance for oxidation to occur. The major disadvantage of preparative HPLC is the smaller sample application

Synthesis and purification of nitrosated catechins

size onto a preparative HPLC column compared to a preparative TLC plate. As a result preparative HPLC is generally more labour intensive than preparative TLC.

In view of the oxidisibility of NCs and the problems experienced in its extraction from silica, preparative HPLC was concluded to be a more appropriate method for large scale purification of NCs. Application of the preparative HPLC method in the present study resulted in successful purification of a mixture of NCs, which enabled further investigations into their biological properties.

CHAPTER FIVE

Absorption and permeability of procyanidins and flavonoids: Caco-2 model

5.1 OBJECTIVES

Chapter 3 described the ability of monomeric and dimeric procyanidins to inhibit tyrosine nitration mediated by nitrite in acid, akin to conditions in the stomach. The procyanidins studied offer protection against RNS derived from acidic nitrite via a mechanism of competitive nitrosation. This prompts the next question as to whether the resulting nitrosated procyanidins are absorbed by the small intestine, which would subsequently affect their biological effects on the human body.

This chapter describes the work undertaken to investigate the absorption and metabolism of nitrosated catechins using the *in vitro* Caco-2 permeability model. The main objectives were:

1) To investigate the apical to basolateral permeability of 3-nitrotyrosine.

2) To investigate the apical to basolateral permeability of nitrosated catechins in comparison with the permeability of other flavonoids.

3) To determine the basolateral to apical permeability of nitrosated catechin and several other flavonoids.

   By determining the basolateral to apical permeability of compounds and the comparison with the apical to basolateral permeability, information can be obtained as to whether the compounds are substrates of efflux pumps.

2) To investigate the effect of flavonoids on P-glycoprotein activity.

5.2 RESULTS

5.2.1 Apical to basolateral permeability of 3-nitrotyrosine

The absorption of 3-nitrotyrosine was studied using Caco-2 monolayers grown on polycarbonate filters as a model for the human small intestine. Caco-2 cells were derived from a human colonic adenocarcinoma (Fogh 1977). When cells are cultured for 21 days they undergo both morphological and biochemical differentiation to resemble normal, differentiated enterocytes (Artursson 1990, Hidalgo *et al.,* 1989, Li 2001, Meunier *et al.,* 1995, Pinto 1983).

The permeability of 3-nitrotyrosine was investigated by addition of the test compound to the apical well, which represents the lumen of the small intestine. After 60 min incubation, samples were collected from both apical and basolateral wells and analysed by reverse phase HPLC for the presence of 3-nitrotyrosine. As shown in Fig 5.1 permeability of 3-nitrotyrosine from the apical to basolateral well was observed. The RT of 3-nitrotyrosine was 25.3 min, and was identified by its UV spectrum as compared with that to a known standard. 4-hydroxy,3-nitrophenylacetic acid (NHPA) was used as external standard in all HPLC analysis. However, since NHPA is metabolite of 3-nitrotyrosine, which may be formed upon uptake across the small intestine, the use of NHPA would interfere with sample quantification. Another external standard would be recommended for future studies.

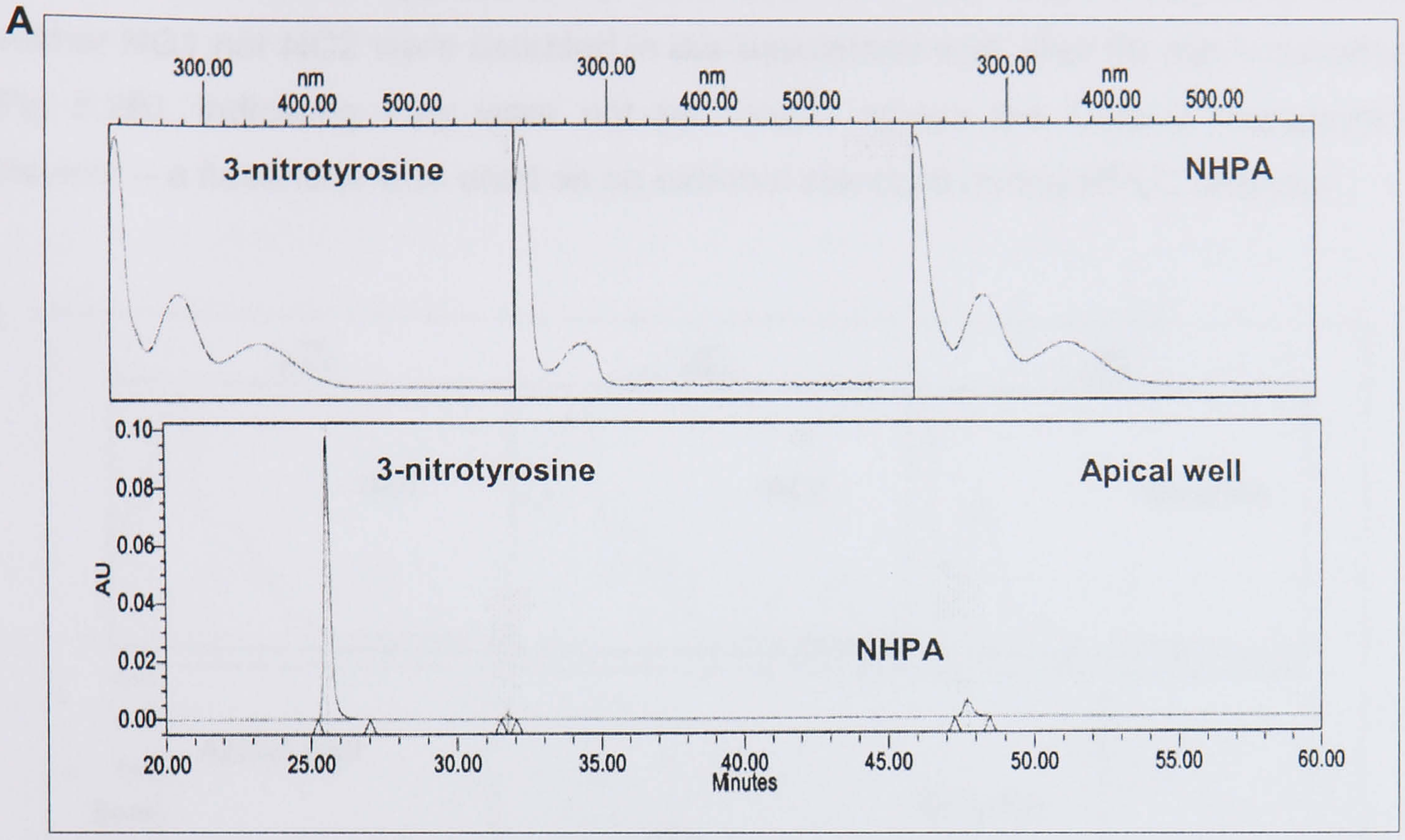
Permeability of 3-nitrotyrosine across the Caco-2 monolayer was expressed as apparent permeability (P_{app}), which was calculated using the following equation:

$P_{app} = V_D / (A.M_D) \times (\Delta M_R / \Delta t)$

Where V_D is the volume of buffer in the donor compartment (cm^3), A is the surface area of the membrane (cm^2), $\Delta M_R / \Delta t$ is the amount of compound transferred to the receiver compartment over time (sec).

The P_{app} of 3-nitrotyrosine was estimated to be $0.41 \pm 0.05 \times 10^{-6}$ cm/sec. No metabolites were detected by HPLC in either the apical or basolateral wells. [^{14}C]-mannitol permeate cells via the paracellular route i.e. between cells, and was therefore used as a marker of paracellular permeability to ensure membrane integrity (see section 2.2.3.2). The P_{app} of [^{14}C]-mannitol was calculated to be $0.53 \pm 0.07 \times 10^{-6}$ cm/sec, which is in agreement with the previously published values

(Vaidyanathan and Walle 2003, Vaidyanathan and Walle 2001, Walgren *et al.*, 1998, Walle *et al.*, 1999).



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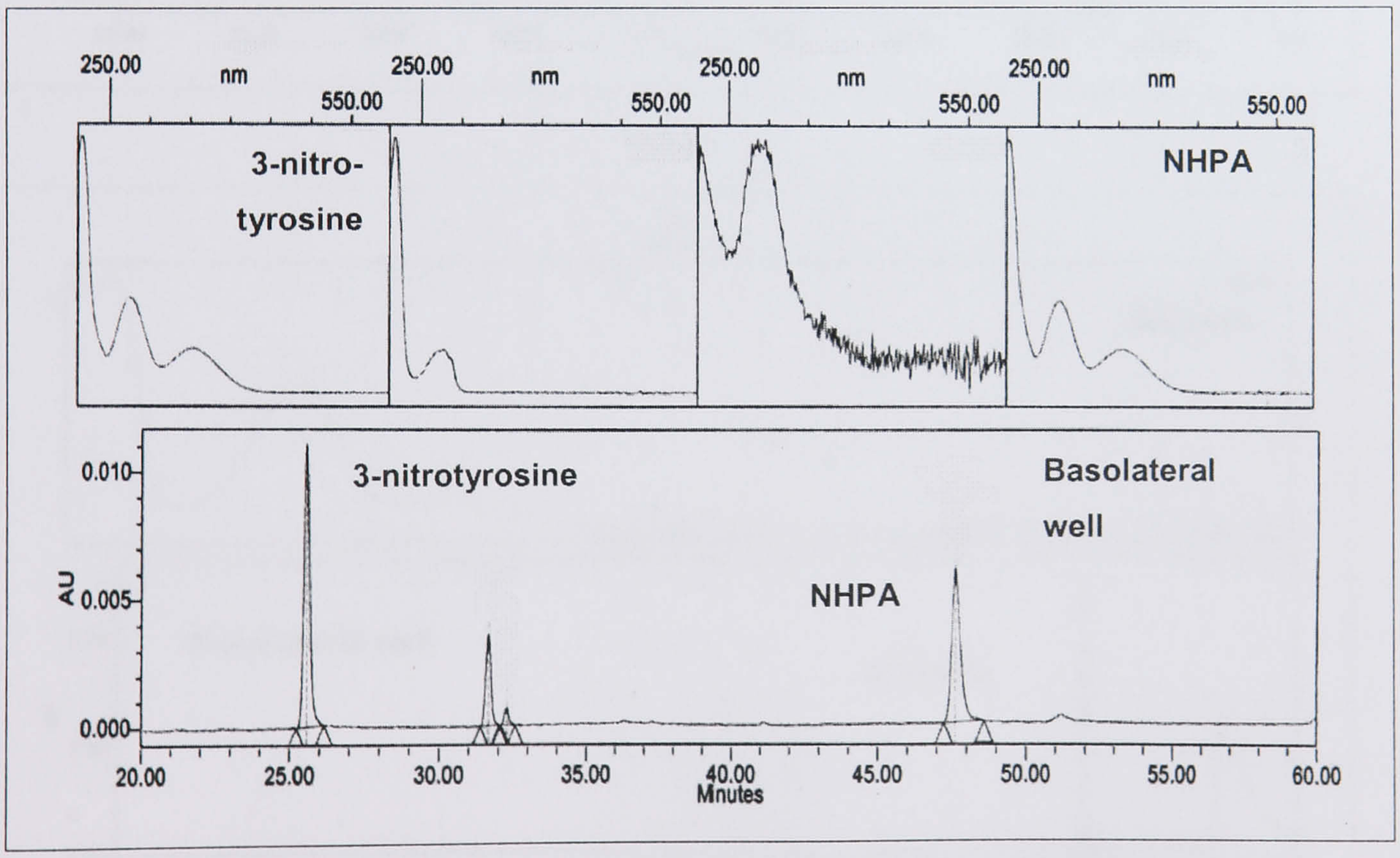


Fig 5.1 Apical to basolateral permeability of 3-nitrotyrosine. HPLC analysis of (A) apical well; (B) basolateral well after 60 min incubation. See section 2.2.3.3 for details on HPLC analysis.

Absorption and permeability of procyanidin and flavonoids: Caco-2 model

5.2.2 *Apical to basolateral permeability of nitrosated catechins*

The apical to basolateral permeability of nitrosated catechins was assessed as described for 3-nitrotyrosine, and the results are depicted in Fig 5.2. The RT's of NC1 and NC2 were approximately 32.9 and 36.4 min respectively (Fig 5.2A). Neither NC1 nor NC2 were detected in the basolateral well after 60 min incubation. (Fig 5.2B), indicating they were not permeable across the Caco-2 monolayers. Didymin – a flavanone was used as an external standard during HPLC analysis.

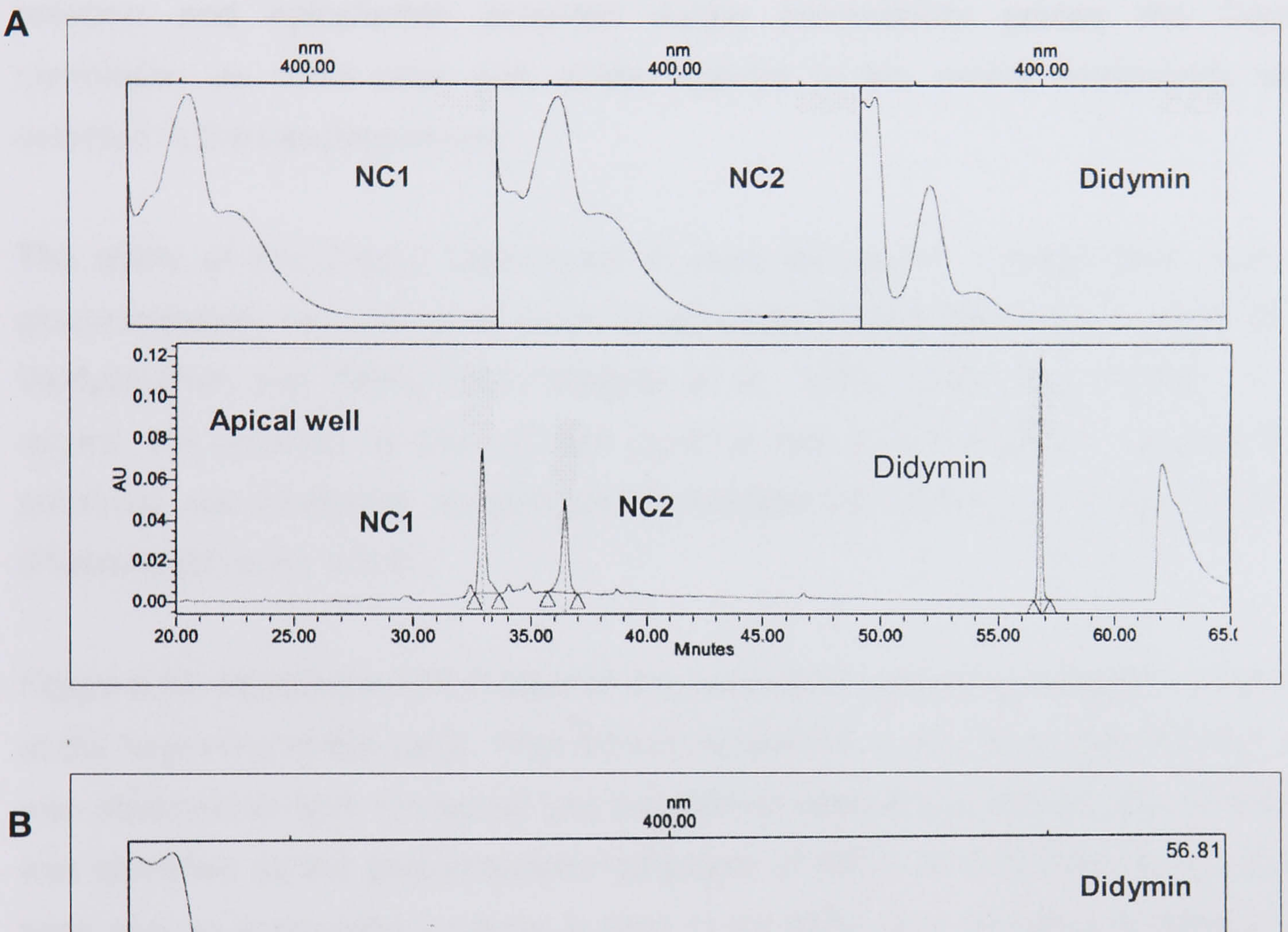


Fig 5.2 Apical to basolateral permeability of NCs. HPLC analysis of (A) apical well; (B) basolateral well after 60 min incubation. See section 2.2.3.3 for details on HPLC analysis.

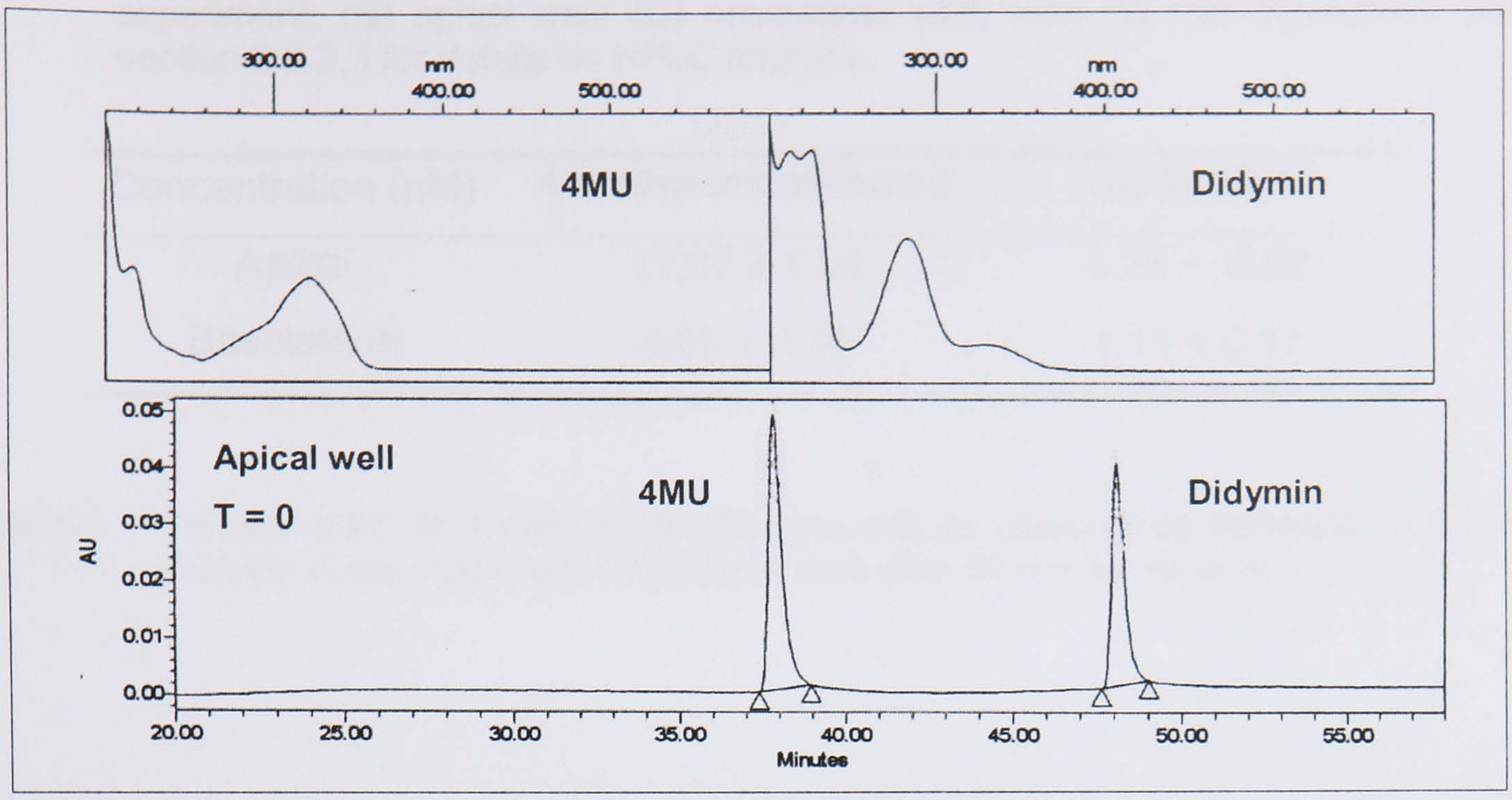
5.2.3 Apical to basolateral permeability of other flavonoids

The A to B permeability of other flavonoids, in particular catechin and epicatechin, were investigated for comparison with NCs. In contrast to NCs, permeability of catechin and epicatechin was observed across the Caco-2 monolayers. No significant difference was found between the P_{app}s of catechin and epicatechin, which were determined to be 0.43 ± 0.11 x 10^{-6} and 0.36 ± 0.15 x 10^{-6} cm/sec, respectively (P>0.05). Some evidence was obtained to suggest metabolism of catechin and epicatechin occurred during permeability across the Caco-2 monolayer, as small peak with similar spectra to the parent compounds were detected in the basolateral well.

The ability of the Caco-2 monolayers to carry out phase II metabolism, such as glucuronidation, has previously been demonstrated (Vaidyanathan and Walle 2003, Vaidyanathan and Walle 2001, Walgren *et al.,* 1998, Walle *et al.,* 1999). In this regard, the potential of Caco-2 cells (used in the current study) to glucuronidate substrate was evaluated, using 4-methylumbelliferone (4MU) as a model substrate (Pissowotzki *et al.,* 2003).

Figure 5.3A depicts the HPLC trace of the apical well containing 4MU (RT~37.8 min) at the beginning of the study. After 60 min incubation, a new peak with RT 27.1 min was observed in both the apical and basolateral wells (Fig 5.3B&C). The new peak was identified as the glucuronidated conjugate of 4MU, as indicated by loss of this peak and an associated increase in area of the 4MU peak following treatment with β-glucuronidase. Further confirmation was obtained by comparing the RT and UV-spectrum of the new peak with a commercially available standard of 4-methylumbeiliferone glucuronide (4MUG). The concentration of 4MU and its glucuronide conjugate in the apical and basolateral wells after 60 min incubation are reported in Table 5.1. The concentration of glucuronide conjugate present in the apical well was higher than in the basolateral well, indicating involvement of efflux pump to transport the glucuronide conjugate out of the cells to the apical side. The P_{app} of 4MU was calculated based on the amount of the intact compound detected in the basolateral well, not the total amount including the glucuronide conjugate. This was found to be 5.91 ± 0.23 x 10^{-6} cm/sec.

A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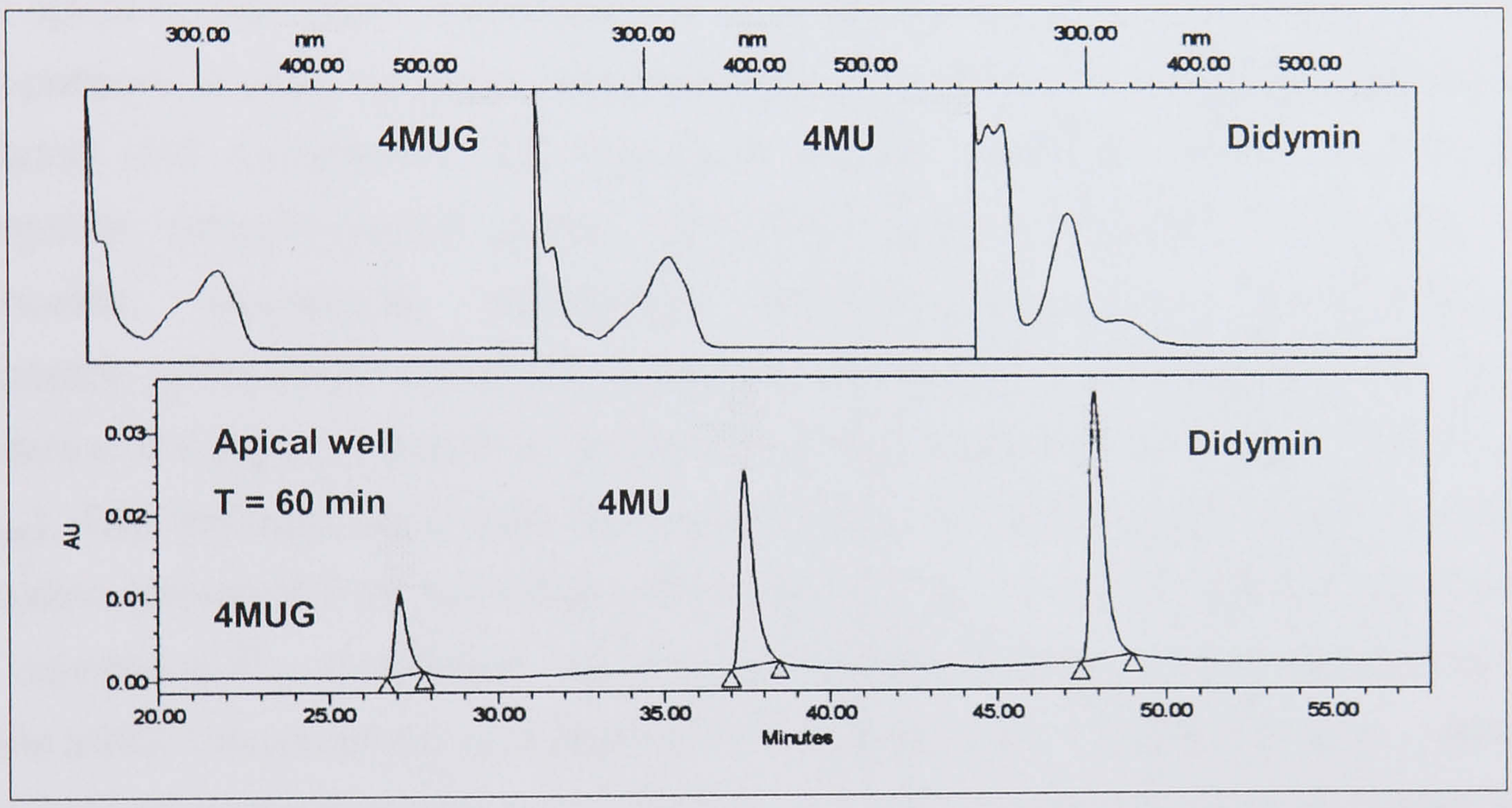
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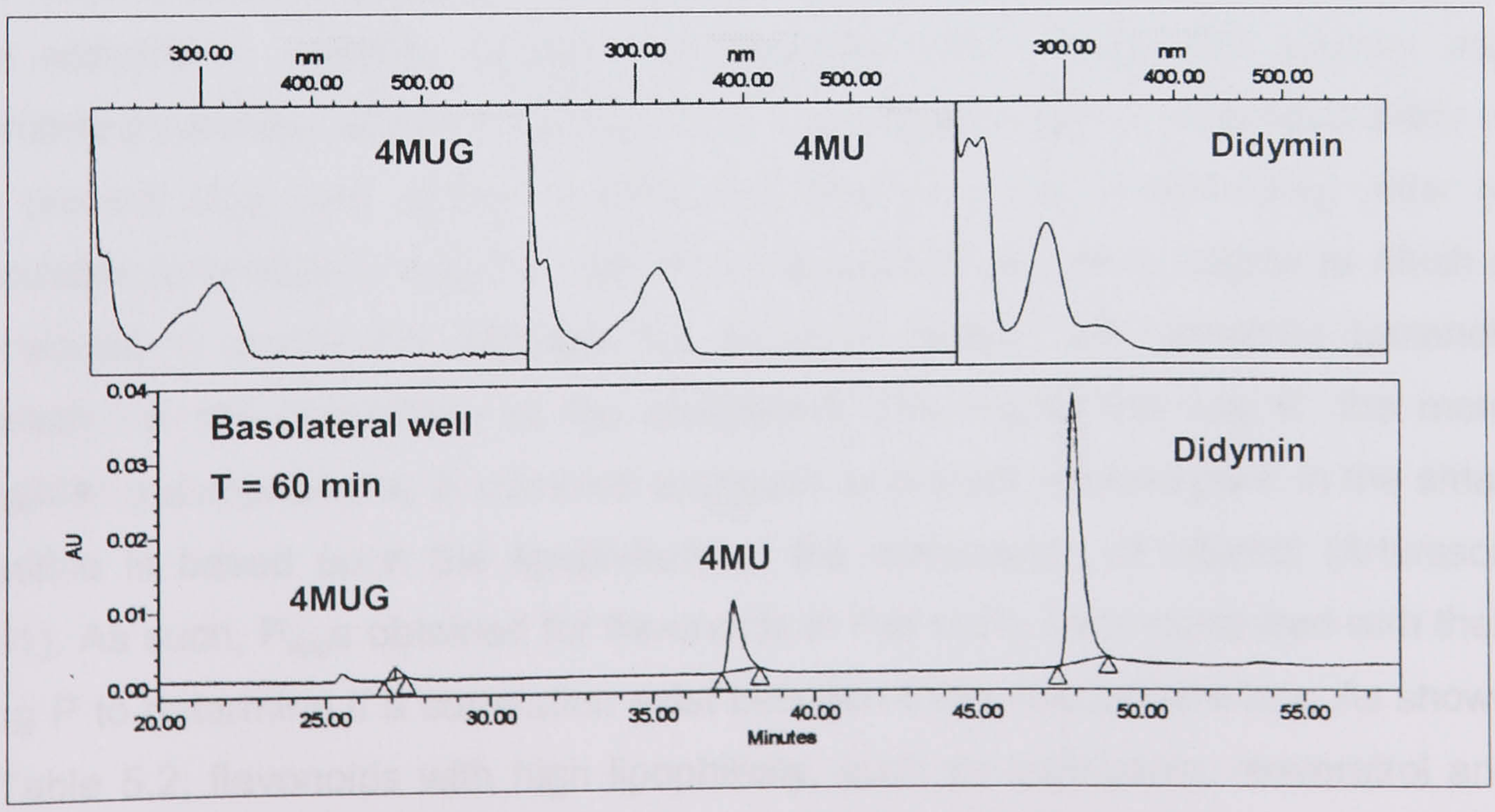
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Fig 5.3 Apical to basolateral permeability of 4MU. HPLC analysis of (A) Start of experiment; (B) apical well; (C) basolateral well, after 60 min incubation. See section 2.2.3.3 for details on HPLC analysis.

Concentration (μM)	4-methylumbelliferone	Glucuronide
Apical	11.07 ± 1.06	4.25 ± 0.42
Basolateral	4.65 ± 0.30	1.11 ± 0.17

Table 5.1 Concentration of 4-methylumbelliferone and its glucuronide detected by HPLC analysis in the apical and basolateral well after 60 min incubation.

The apical to basolateral permeability of other flavonoids were also studied, to allow comparison of their apparent permeabilities with those of nitrosated catechins, catechin and epicatechin. The flavonoids studied included: chrysin, cyanidin-3-rutinoside, epigallocatechin gallate, epicatechin gallate, hesperetin, hesperetin-7-rutinoside, kaempferol, naringenin, naringenin-7-glucoside, pelargonidin-3-glucoside, procyanidin dimer B2, quercetin, resveratrol and rosmarinic acid. The measure of drug permeability is referred to as the apparent permeability coefficient (P_{app}). Two methods were used for calculating P_{app}s in the present study. The first equation (equation 2 as described in Section 2.2.3.4) is commonly used in literature for calculating P_{app} of different compounds including flavonoids (Vaidyanathan and Walle 2003, Vaidyanathan and Walle 2001, Walgren *et al.,* 1998, Walle *et al.,* 1999). The second method (equation 3 in Section 2.2.3.4) follows the same principles as that adopted in equation 2, with the exception that it takes into account any membrane retention of the compound. The P_{app}s determined for these flavonoids in the present study are shown in Table 5.2, and are listed in ascending order of calculated lipophilicity (cLog P). Log P is a measurement of the degree to which a compound is partitioned between an aqueous (water) and lipophilic (octanol) medium i.e. the lipophilicity of the compound. The higher the Log P, the more lipophilic a compound is. A common approach to predicting absorption in the small intestine is based upon the lipophilicity of the compounds of interest (Artursson 1991). As such, P_{app}s obtained for flavonoids in this study were compared with their cLog P to determine if a correlation exist between these two parameters. As shown in Table 5.2, flavonoids with high lipophilicity, such as naringenin, resveratrol and chrysin were found to have high P_{app}s compared to other flavonoids. The relationships between cLog P of flavonoids and their P_{app}s is depicted in Fig 5.4.

Absorption and permeability of procyanidin and flavonoids: Caco-2 model

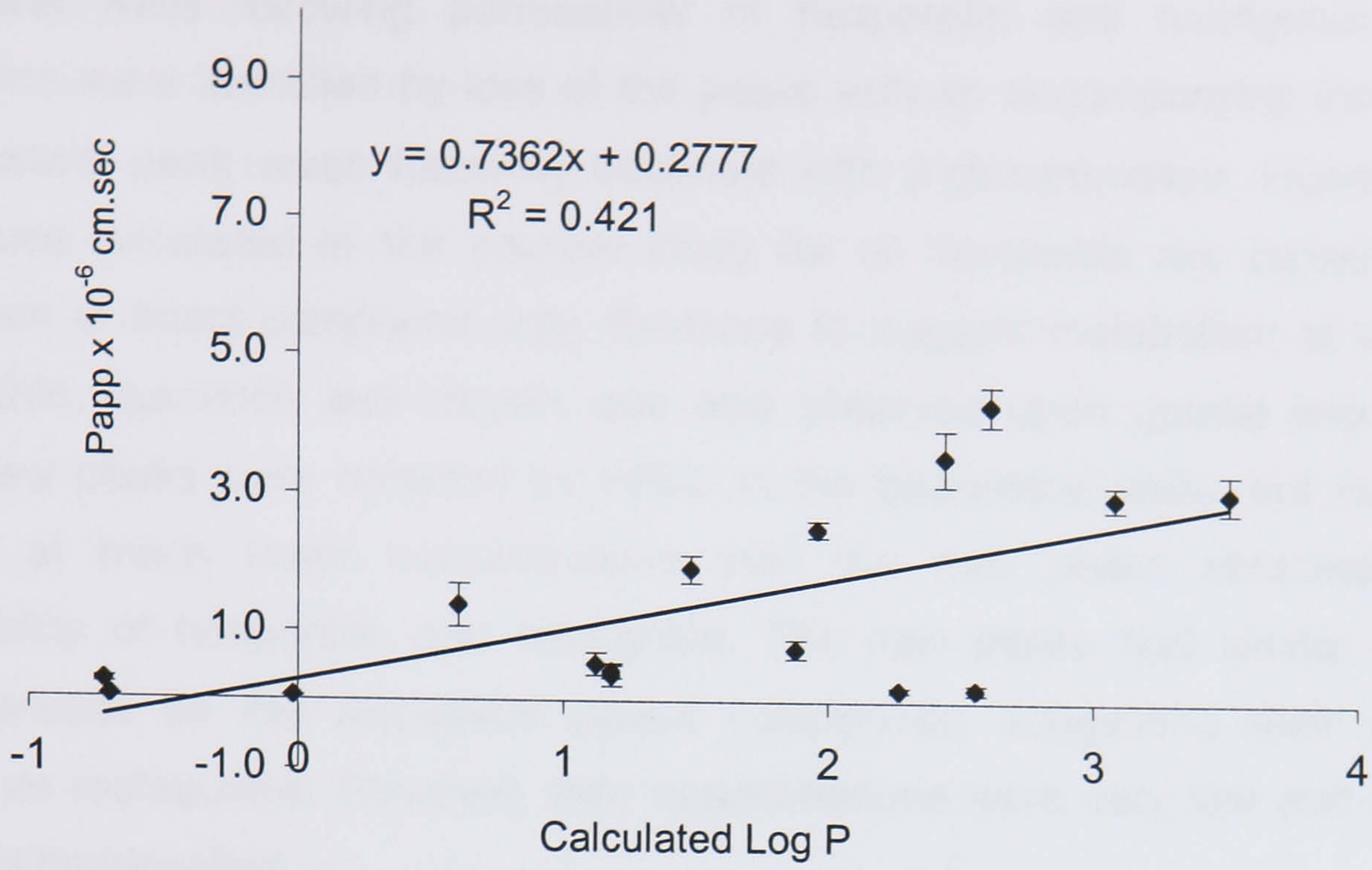
Flavonoids	*Log P*	*P_{app} x 10^{-6} (cm/sec) (mean ± SEM)*	
		Equation 1	*Equation 2*
Hesperedin	-0.72	0.26 ± 0.04	0.56 ± 0.04
Cyanidin-3-rutinoside	-0.7	0.04 ± 0.001	0.04 ± 0.001
Pelargonidin-3-glucoside	-0.02	0.05 ± 0.004	0.06 ± 0.004
Naringenin-7-glucoside	0.6	1.37 ± 0.31	1.91 ± 0.33
Epicatechin gallate	1.12	0.53 ± 0.17	0.55 ± 0.17
Epicatechin	1.18	0.37 ± 0.15	0.33 ± 0.17
Catechin	1.18	0.43 ± 0.11	0.45 ± 0.12
Quercetin	1.48	1.91 ± 0.20	2.76 ± 0.23
Procyanidin dimer B2	1.88	0.76 ± 0.13	0.78 ± 0.14
Kaempferol	1.96	2.50 ± 0.12	3.52 ± 0.15
Rosmarinic acid	2.27	0.17 ± 0.04	0.46 ± 0.04
Hesperetin	2.44	3.55 ± 0.41	4.90 ± 0.52
Epigallocatechin gallate	2.56	0.19 ± 0.07	0.29 ± 0.07
Naringenin	2.61	4.31 ± 0.29	8.15 ± 0.40
Resveratrol	3.08	2.97 ± 0.18	5.76 ± 0.22
Chrysin	3.51	3.04 ± 0.28	4.35 ± 0.36

Table 5.2 Apical to basolateral permeability (P_{app}) of flavonoids calculated using equation 1 which is commonly used in literature, and equation 2 with correction for membrane retention. Each value represents the mean ± SEM of 4-10 samples.

As shown in Fig 5.4A a weak correlation (R^2 = 0.42) was observed between the cLog P and P_{app}s of flavonoids calculated using the standard equation (equation 2) routinely used in literature. This suggests that the permeability of flavonoids across Caco-2 monolayer is only weakly correlated with their lipophilicity, and that other factors are likely to be involved in the transport of flavonoids. When equation 3 was applied to calculate P_{app}s of the flavonoids, taking in account any membrane retention, the correlation between cLog P and P_{app} remained unchanged (R^2 = 0.40).

Absorption and permeability of procyanidin and flavonoids: Caco-2 model

A **Log P vs. P_{app} (equation 1)**



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Log P vs. P_{app} (equation 2)

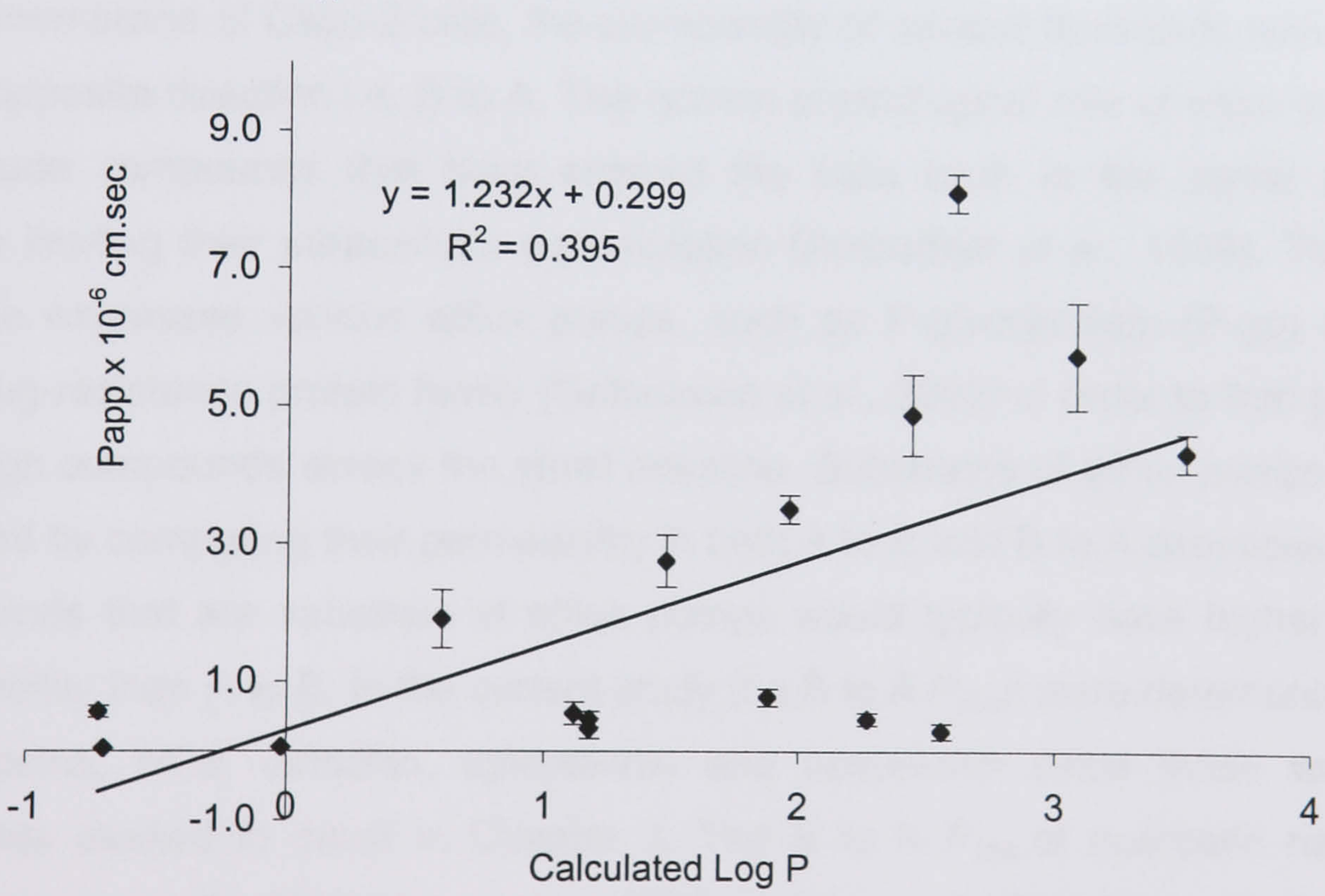


Fig 5.4 Correlation between the apparent permeability (P_{app}) of flavonoids determined using the Caco-2 permeability model with their calculated Log P. (A) P_{app}s calculated using equation 2; (B) P_{app}s calculated using equation 3.

Evidence of metabolism upon absorption across the Caco-2 monolayer was also observed for several flavonoids. Glucuronide conjugates were measured in the basolateral wells following permeability of hesperetin and naringenin. These conjugates were identified by loss of the peaks with an accompanying increase in the aglycone peak areas following treatment with β-glucuronidase. However, the P_{app} values calculated in the present study for all flavonoids are based on the absorption of intact compound only. Evidence to suggest metabolism of catechin, epicatechin, quercetin and chrysin was also observed upon uptake into Caco-2 cells. New peaks were detected by HPLC in the basolateral wells, but they were present at much lower concentrations than the new peaks obtained during permeability of hesperetin and naringenin. The new peaks had similar spectral characteristics as the respective parent compounds, suggesting their possible identity as metabolites. However, their concentrations were very low and as such could not be identified.

5.2.4 Basolateral to apical permeability of other flavonoids

In order to study whether flavonoids are substrates for efflux pumps present on the apical membrane of Caco-2 cells, the permeability of several flavonoids was studied in the opposite direction i.e. B to A. The normal physiological role of efflux pumps is to extrude compounds that have entered the cells back to the apical surface, thereby limiting their intracellular accumulation (Ambudkar *et al.,* 1999). The small intestine expresses various efflux pumps, such as P-glycoprotein (P-gp) and the multidrug-resistance protein family (Gottesman *et al.,* 2002) in order to limit passage of foreign compounds across the small intestine. Substrates of efflux pumps can be identified by comparing their permeability in both A to B and B to A directions. Those compounds that are substrate of efflux pumps would typically have higher B to A permeability than A to B. In the current study the B to A P_{app}s were determined for 3-nitrotyrosine, NCs, catechin, epicatechin and hesperetin since these were the flavonoids studied in detail in Chapter 3. The B to A P_{app} of quercetin has been reported previously (Walgren *et al.,* 1998), and was studied here as a positive control.

The B to A P_{app} of 3-nitrotyrosine was calculated to be $4.18 \pm 0.36 \times 10^{-6}$ cm/sec, which was ten fold higher than the A to B P_{app} (Table 5.3). No metabolites of 3-nitrotyrosine were observed during B to A permeability studies in either the apical or basolateral wells. Quercetin and hesperetin also demonstrated B to A permeability.

Absorption and permeability of procyanidin and flavonoids: Caco-2 model

In the case of the flavanol quercetin the B to A permeability was 3-fold greater compared with its A to B permeability ($P<0.001$). Hesperetin showed the highest B to A permeability of the different flavonoids studied. Like quercetin this value was approximately 3-fold higher than its permeability observed from A to B ($P<0.001$). By contrast catechin did not demonstrate any measurable B to A permeability in the current series of experiments, despite showing A to B permeability (Table 5.3). Several peaks with similar spectral characteristic to catechin were detected in apical and basolateral wells during B to A studies. These were suggested to be metabolites of catechin although identification using mass spectrometry was unsuccessful due to their low concentration. In addition, it has previously been reported in section 5.2.2 that NCs did not demonstrate measurable A to B permeability across the Caco-2 monolayer. This property was also observed when examining their B to A permeability over 60 min (Table 5.3). The greater flux of compounds from B to A compared with their A to B fluxes suggests that their permeability across the monolayer is mediated by the role of efflux pumps on the apical membrane surface.

Flavonoids	*P_{app} x 10^{-6} (cm/sec) (mean ± SEM)*	
	A to B	*B to A*
3-Nitrotyrosine	0.41 ± 0.05	4.18 ± 0.36***
Nitrosated catechins	ND	ND
Epicatechin	0.37 ± 0.15	0.97 ± 0.11*
Catechin	0.43 ± 0.11	ND
Quercetin	1.91 ± 0.20	5.93 ± 0.23***
Hesperetin	3.55 ± 0.41	9.71 ± 0.39***

Table 5.3 A comparison of the apical to basolateral and basolateral to apical permeabilities (P_{app}) of 3-nitrotyrosine, NCs, epicatechin, quercetin and hesperetin calculated using equation 2. Data are expressed as the means ± SEM of at least 5 determinations. Difference in A to B and B to A P_{app} of each compound was evaluated using unpaired, two-tailed Student's t-test. ***, * represent significantly greater B to A P_{app}s than A to B at $P<0.001$ and $P<0.05$, respectively. ND; not detected.

Absorption and permeability of procyanidin and flavonoids: Caco-2 model

5.2.5 Interaction with P-glycoprotein

The previous section suggests that the permeability of epicatechin, quercetin and hesperetin were influenced by an efflux mechanism present on the apical membrane of Caco-2 cells. However, it was not determined which efflux pump was responsible for this phenomenon. In this section the interaction of catechin, epicatechin, quercetin and hesperetin with P-gp was determined using Madin-Darby Canine Kidney cells with high P-gp expression through transfection of cells with the full length cDNA for human multidrug resistance protein 1 (MDCK-MDR1 cells) (Pastan *et al.,* 1988). Different inhibitors of P-gp were examined to identify a suitable positive control for subsequent experiments. This was accomplished by studying their effects on the accumulation of Rhodamine 123 (R123) (a fluorescence substrate of P-gp) in MDCK-MDR1 cells, a common method for evaluating the functional activity of P-gp in cells (Batrakova *et al.,* 2003, Fontaine *et al.,* 1996).

Figure 5.5 depicts the effect of known P-gp inhibitors: GF120918 (Seral *et al.,* 2003), PSC833, cyclosporin A and verapamil (van der Sandt *et al.,* 2000), on R123 uptake in MDCK-MDR1 cells. All four compounds tested were confirmed to be inhibitors of P-gp activity, as demonstrated by increased R123 accumulation in MDCK-MDR1 cells. Using R123 at 10 μM, cyclosporin A (50 μM) was found to be the most effective, inhibiting P-gp mediated efflux by 80.2 ± 6.0% ($P<0.001$ compared with R123 accumulation in the absence of inhibitor), followed by PSC833 and GF120918 which inhibited efflux by 63.4 ± 6.6 and 38.0 ± 6.3% respectively ($P<0.001$ in each case). The least effective inhibitor amongst those tested was verapamil, showing just 13.0 ± 1.4% inhibition of R123 (10 μM) efflux. A similar pattern on inhibitory potency was observed using R123 at 20 μM (Fig 5.5). Cyclosporin A was the most effective inhibitor (102.1 ± 4.5%; $P<0.001$), while verapamil was the least effective inhibitor (35.8 ± 2.8%; $P<0.001$) of P-gp activity. A similar inhibitory potential was observed between PSC833 and GF120918, both displaying ~69% inhibition ($P<0.001$ in each case). In summary, the order of effectiveness in the inhibition of P-gp activity is cyclosporin A > PSC833 > GF120918 > verapamil at 10 μM R123, and cyclosporin A > PSC833 ≥ GF120918 > verapamil at 20 μM R123. Since cyclosporin A was found to be the most effective inhibitor of R123 accumulation in MDCK-MDR1 cells, it was chosen as a positive control in all subsequent experiments.

Absorption and permeability of procyanidin and flavonoids: Caco-2 model

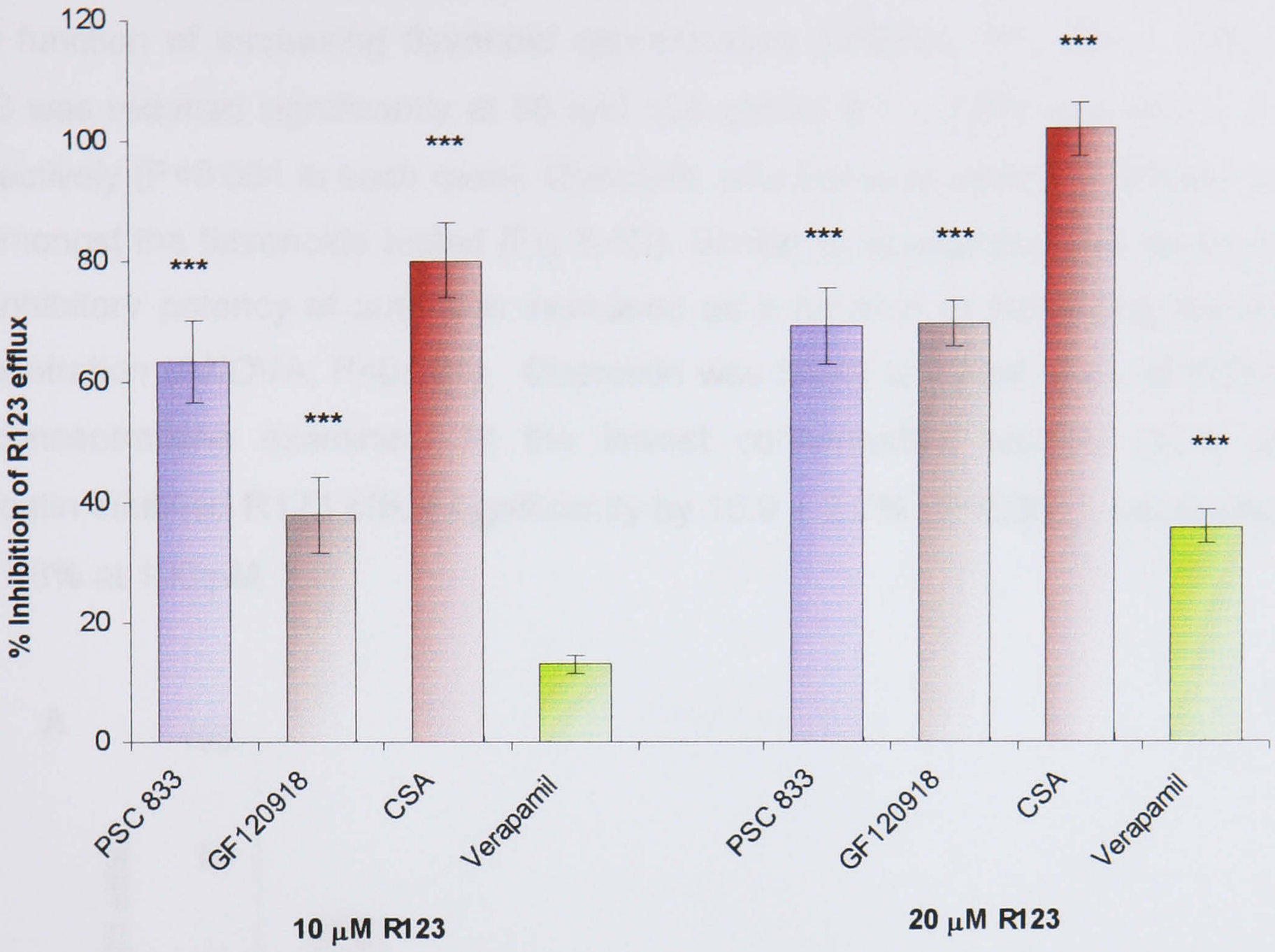
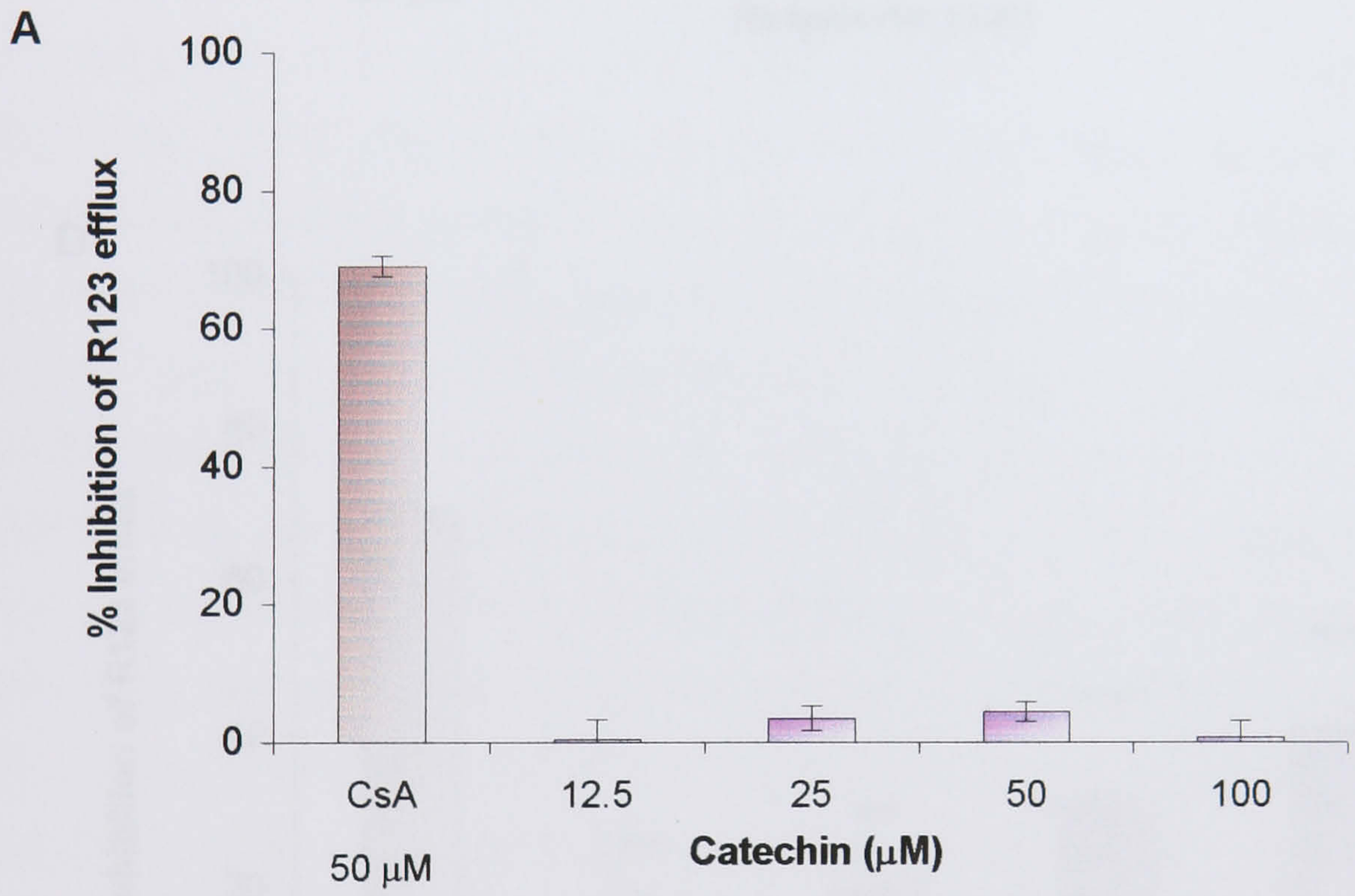


Fig 5.5 Percentage Inhibition of R123 efflux mediated by P-gp in the presence of 50 μM PSC833, GF120918, cyclosporin A and verapamil. Data are expressed as means ± SEM of eight determinations. Statistical analysis was carried out by comparing the % inhibition of each modulator with the control using unpaired, two-tailed Student's t-test. With the exception of 10 μM verapamil, the presence of all modulators led to a significant inhibition of R123 efflux at both concentrations by P-gp in MDCK-MDR1 cells compared with control cells. ***P < 0.001; and **P < 0.01.

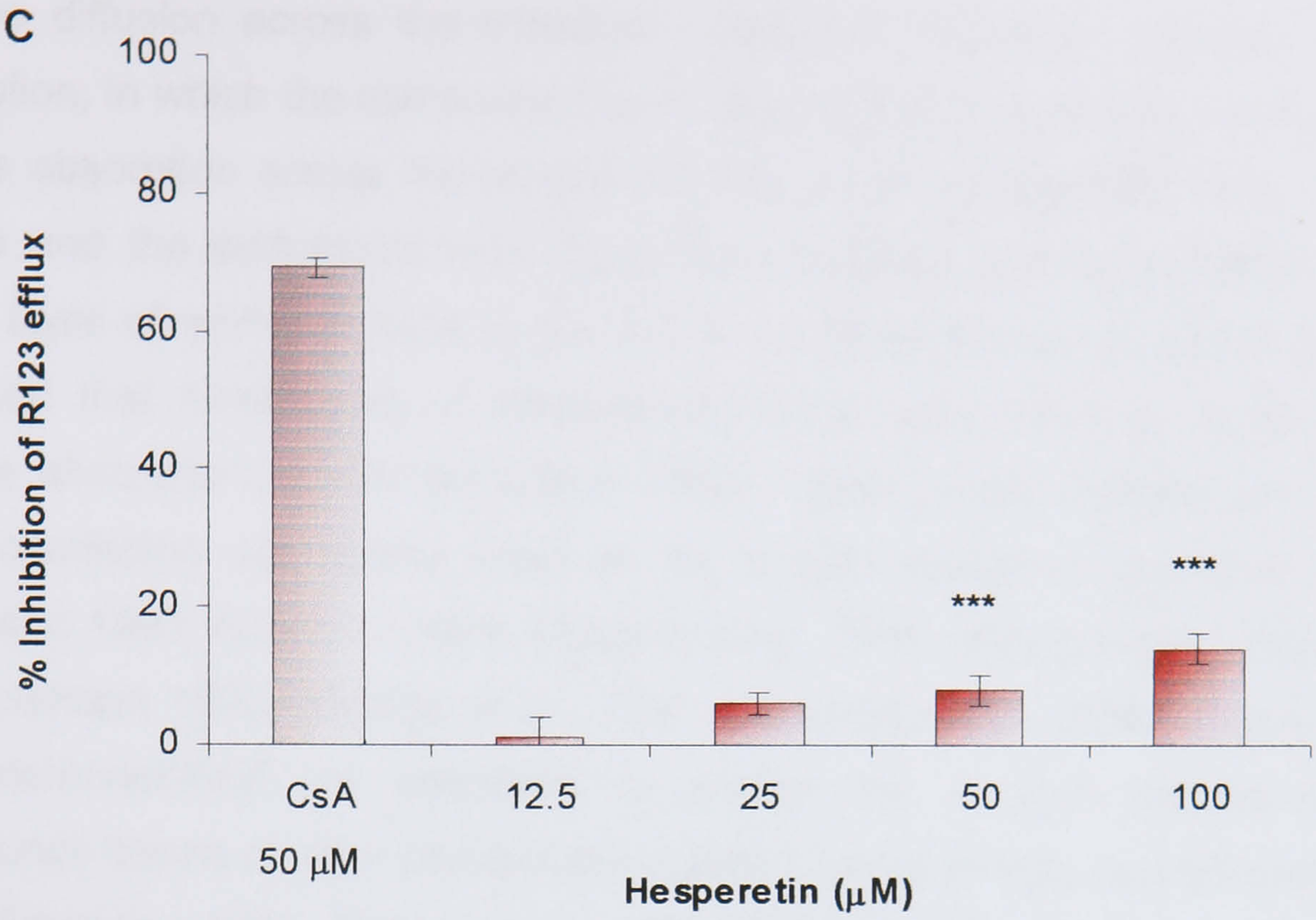
To determine the ability of catechin, epicatechin, quercetin and hesperetin to inhibit the P-gp efflux activity, accumulation of R123 was studied in MDCK-MDR1 cells over a flavonoid concentration range 12.5 to 100 μM. The results are depicted in Fig 5.6. Cyclosporin A (50 μM) was used as a positive control for the inhibition of P-gp activity, displayed 63.2 ± 2.8% inhibition. No inhibition of P-gp activity was observed with catechin at any concentration tested, suggesting a lack of interaction between catechin and P-gp over this concentration range (Fig 5.6A). In contrast, epicatechin demonstrated significant inhibition of R123 efflux as a function of increasing concentration (ANOVA; P<0.001) (Fig 5.6B). In comparison with R123 accumulation in cells in the absence of epicatechin, a significant inhibitory effect was observed with epicatechin at 25 μM (P<0.01); 50 μM (P<0.001) and 100 μM (P<0.001). The

Absorption and permeability of procyanidin and flavonoids: Caco-2 model

highest inhibition of P-gp activity was observed at 100 μM epicatechin (10.9 ± 1.6%). A similar pattern of inhibition was seen with hesperetin (Fig 5.6C), increasing as a function of increasing flavonoid concentration (ANOVA; P<0.001). Efflux of R123 was reduced significantly at 50 and 100 μM by 8.1 ± 2.3% and 14.0 ± 2.2% respectively (P<0.001 in each case). Quercetin was the most effective inhibitor of P-gp amongst the flavonoids tested (Fig 5.6D). Similar to epicatechin and hesperetin, the inhibitory potency of quercetin increased as a function of increasing flavonoid concentration (ANOVA; P<0.001). Quercetin was found to inhibit efflux of R123 at all concentrations examined. At the lowest concentration studied (12.5 μM), quercetin inhibited R123 efflux significantly by 16.9 ± 2.7% (P<0.001), increasing to 47 ±1.8% at 100 μM.



Absorption and permeability of procyanidin and flavonoids: Caco-2 model



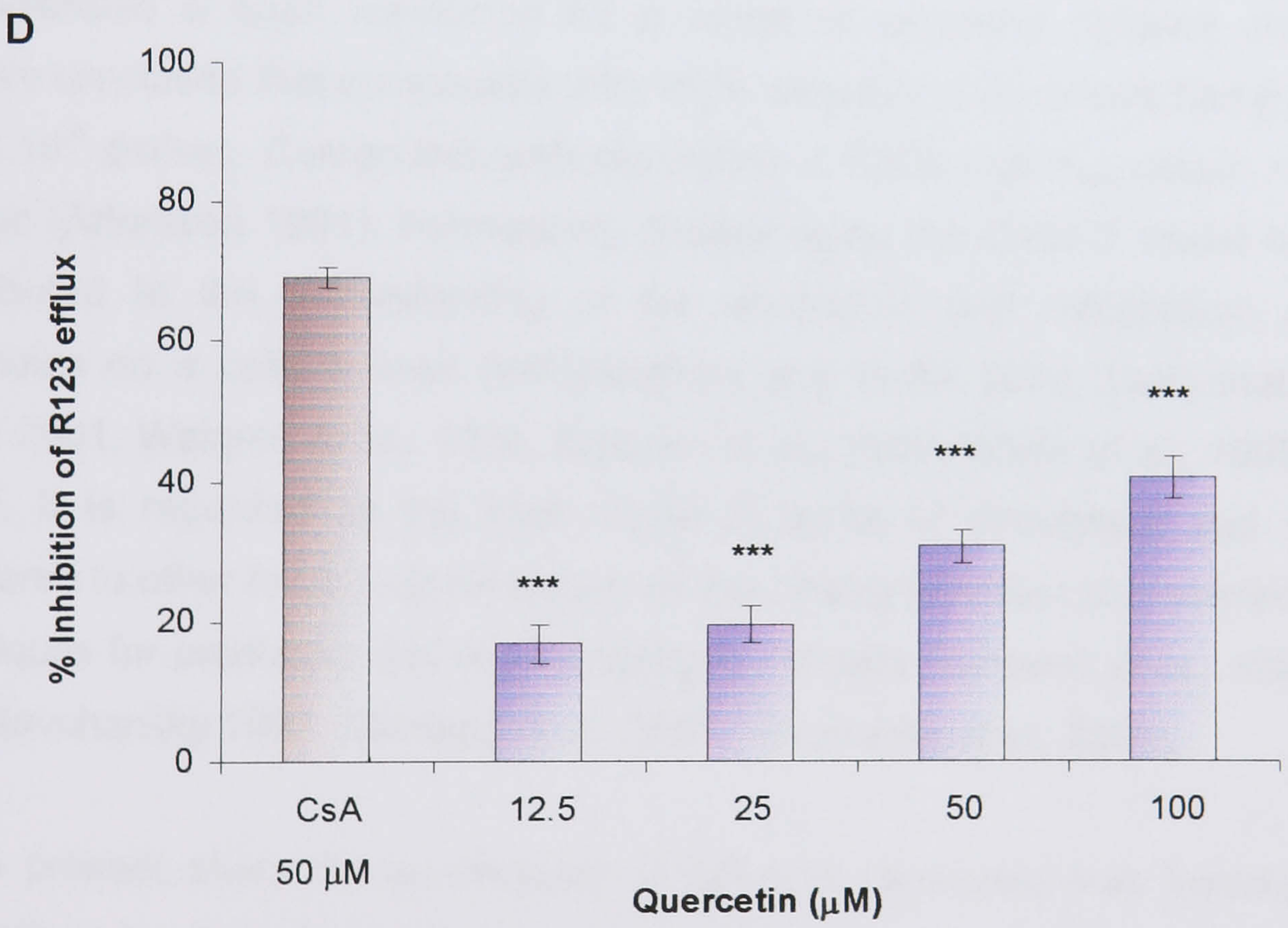


Fig 5.6 Percentage inhibition of R123 efflux mediated by P-gp in the presence of catechin, epicatechin, hesperetin and quercetin at 12.5 to 100 μM. Data are expressed as means ± SEM of 16 determinations. Statistical analysis was carried out by comparing all columns with treatment of flavonoids to control cells using one-way ANOVA with Tukey-Kramer multiple comparisons post *hoc* tests, ***P < 0.001; *P < 0.05. Apart from catechin, all flavonoids at 50 μM or above exhibited significant inhibition of R123 efflux by P-gp in MDCK-MDR1 cells compared to control cells.

Absorption and permeability of procyanidin and flavonoids: Caco-2 model

5.3 DISCUSSION

Passive diffusion across the intestinal mucosa is the most common method of absorption, in which the compound has to diffuse across a series of barriers. These include absorption across the mucus gel, the intestinal epithelial cells, the lamina propria and the endothelial cells lining the capillaries (Jackson 1987). Since the single layer of epithelial cells is the most restrictive barrier to absorption, it was proposed that monolayers of intestinal epithelial cells could be used to predict passive absorption *in vivo* (Artursson 1991). Caco-2 cells derived from a human colon carcinoma are widely used as an *in vitro* model of intestinal epithelium (Artursson 1990, Artursson 1991, Caldwell *et al.,* 1998, Hidalgo *et al.,* 1989, Hidalgo and Borchardt 1990, Hidalgo *et al.,* 1991, Hochman *et al.,* 2000). Several reports have demonstrated the possibility to predict the *in vivo* oral absorption of compounds based on their permeability across Caco-2 monolayers (Artursson *et al.,* 2001, Artursson 1991, Rubas *et al.,* 1993, Stewart *et al.,* 1995, Yamashita *et al.,* 1997, Yee 1997). Early work by Artursson and Karlsson (Artursson 1991) demonstrated a good correlation for a range of passively diffused drugs. The authors concluded that compounds with 100% absorption in humans had P_{app} values > 1 x 10^{-6} cm/sec. Compounds with absorption < 100% had P_{app} values < 1 x 10^{-6} cm/sec (Artursson 1991). Permeability studies using the Caco-2 model have also contributed to the understanding of the absorption and metabolism of many flavonoids on a cellular level (Vaidyanathan and Walle 2003, Vaidyanathan and Walle 2001, Walgren *et al.,* 1998, Walgren *et al.,* 2000, Walle *et al.,* 1999, Walter 1995). It is regarded as the best model in terms of throughput and reliability compared to other *in vitro* systems such as the Ussing chamber and everted gut sac techniques for predicting oral bioavailability in humans (Caldwell *et al.,* 1998, Pade and Stavchansky 1997, Stenberg *et al.,* 2001, Yamashita *et al.,* 2000).

In the present study the permeability of different flavonoids was investigated in comparison to nitrosated catechins. The findings observed in this study show that NC1and NC2 were not permeable across the Caco-2 monolayer, suggesting they cannot be absorbed by the human small intestine. However the remaining flavonoids studied (Table 5.2) exhibited measurable permeabilities across the Caco-2 monolayer, albeit to differing extents. The P_{app}s of quercetin obtained in the present study (A to B: 1.91 ± 0.20 x 10^{-6} and B to A: 5.93 ± 0.23 x 10^{-6} cm/sec) was lower than that reported by Walgren *et al.,* (Walgren *et al.,* 1998) (A to B: 5.8 ± 1.1 x 10^{-6} and B to A: 11.1 ± 1.2 x 10^{-6} cm/sec). In contrast, higher P_{app}s was

demonstrated by epicatechin (A to B: 0.37 ± 0.15 x 10^{-6} and B to A: 0.97 ± 0.11 x 10^{-6} cm/sec) compared to Vaidyanathan *et al.*, (Vaidyanathan and Walle 2001) reporting a B to A P_{app} of 0.67 ± 0.05 x 10^{-6} cm/sec and no permeability in the A to B direction. The difference observed between the P_{app}s of flavonoids obtained in this study and the published values in the literature is probably due to differences in the experimental conditions used. This will be discussed in further details later in this Chapter.

Intestinal absorption of a compound is a complex issue affected by numerous factors, such as compound lipophilicity, solubility, metabolism by enzymes in the intestinal lumen, interaction with active transporters upon absorption as well as interactions with efflux systems. Nevertheless, a common approach to predicting absorption in the small intestine is based upon the lipophilicity of the compounds of interest (Artursson 1991). When the P_{app}s of 20 drugs, with different structural properties was investigated as a function of lipophilicity, a coarse correlation (R^2 = 0.58) was reported (Artursson 1991). When the flavonoid P_{app}s were compared with their respective cLog Ps, a weak correlation was observed (R^2 = 0.42). An issue that is often overlooked in P_{app} calculations using the Caco-2 permeability model is the membrane retention of test compounds. Permeability is usually determined by the appearance of test compounds in the receiver wells. As lipophilic compounds permeate across a cellular membrane, a fraction of the solute can be retained within the lipid bilayer (Youdim 2003). All too often, permeability calculations are based only on the amount of compound present in the receiver wells, neglecting the amount retained in the cell membrane, which may lead to an underestimation of their permeability. In order to address this potential underestimation, calculations were performed that took into account membrane retention (equation 3, section 2.2.3.4). As shown in Table 5.2 there were no significant changes in the permeability values obtained using this correction with that not taking into account membrane retention. Moreover the correlation between these permeability values and the flavonoid cLog Ps was unchanged. Taken together, these observations suggest that the series of flavonoids studied did not exhibit any significant retention within the membrane bilayer during absorption, and that the limited permeabilities measured for certain flavonoids is not solely dependent on their lipophilicities. In this regard, although the cLog P of NC1 and NC2 is 0.92, similar to catechin and epicatechin (Log P = 1.18), NC1 and NC2 were not permeable across the Caco-2 monolayers, while catechin and epicatechin demonstrated P_{app}s of 0.43 ± 0.11 and 0.37 ± 0.15 x

Absorption and permeability of procyanidin and flavonoids: Caco-2 model

10^{-6} cm/sec respectively. It is therefore clear that lipophilicity only gives an indication of drug absorption (Artursson *et al.,* 2001, Barthe *et al.,* 1999, Martin 1981), and that other parameters independent of lipophilicity must also be taken into account, for example the role of active transport systems in mediating the permeability of flavonoids across the Caco-2 monolayer.

There are five mechanisms by which a compound can cross the epithelial cell layer of the small intestine (Fig. 1.5), namely paracellular transport, transcellular transport, carrier-mediated transport, multidrug resistance-mediated efflux and endocytosis. However, the route of flavonoid uptake into cells was not investigated in the present study. Uptake of quercetin has been suggested to occur transcellularly based on the findings that it has a P_{app} value ten times higher than mannitol, which crosses via the paracellular route (Walgren *et al.,* 1998). In the current study, several flavonoids were shown to undergo metabolism during transfer across the Caco-2 monolayer. These included: hesperetin, naringenin, catechin, epicatechin, quercetin and chrysin. Since phase I and II metabolising enzymes are mainly localised in the cytoplasm and endoplasmic reticulum within enterocytes (Table 1.2), these compounds must have been absorbed via the transcellular route in order that they be able to undergo intracellular metabolism. To identify compounds that cross monolayers via the paracellular route (gap between adjacent cells), experiments can be carried out in the absence of extracellular calcium. The P_{app} of compounds absorbed via the paracellular pathway would be expected to increase due to opening of the tight junctions in the absence of calcium (Artursson and Magnusson 1990). Thus the Caco-2 model can be a valuable tool to study mechanisms/pathway of transport on a cellular level. Knowledge about the transport route of different compounds can give insights into the variation in their rate of oral absorption. Because less surface area is available to the molecules during paracellular transport, it is a less efficient process than the transcellular route and lower P_{app}s are often expected (Gan 1997).

Caco-2 monolayers are being used frequently as an *in vitro* model for rapid screening of intestinal absorption owing to their wide application. Apart from being a good model for predicting oral absorption in human, Caco-2 model can be used to study metabolism, interaction with active transporters and efflux pumps in the small intestine. Moreover, information regarding the mode of transport across enterocytes can also be obtained. The drawback of this model is that inconsistencies have been identified in experimental conditions between different laboratories, making it difficult

to compare results obtained from different authors. This section will highlight the importance of identifying conditions to better mimic *in vivo* conditions of the small intestine. In addition, optimised conditions for Caco-2 permeability experiments will be suggested in order to standardise culturing and experimental protocols between different laboratories.

Factors influencing permeability prediction	*Parameter(s) affected*
• Source of cells and passage number	Intactness of monolayer[1], expression of endogenous transporters[2,3]
• Growth medium	Time to confluency, expression of endogenous transporters[4]
• Composition and porosity of filter in transport unit	Adsorption, diffusion of solutes[1,5]
• Length of time cells grown on filters	Maturity of monolayer, tight junctions
• Transport buffer composition	Cost, efficiency, sink condition[6,7], analysis[8]
• pH of transport buffer	Property (ionisation) of test compound[8]
• Time and temperature	Viability of cells
• Mixing or stirring	Unstirred water layer effects[9]

Table 5.4 Factors potentially influencing the prediction of permeability using Caco-2 monolayers as a screening model. [1](Walter 1995); [2](Woodcook *et al.,* 1991), [3](Walter and Kissel 1994); [4] (Ingels and Augustijns 2003); [5](Nicklin *et al.,* 1992); [6](Krishna *et al.,* 2001); [7](Saha and Kou 2002); [8](Yamashita *et al.,* 2000); [9](Naruhashi *et al.,* 2003).

Caco-2 cells are a heterogeneous cell population because of varying culturing conditions used in different laboratories, which causes different selection pressures (Artursson *et al.,* 2001). Striking differences in transepithelial electrical resistance (TEER) (Walter 1995), morphology (Woodcook *et al.,* 1991), density of microvilli (Walter 1995) and expression of active transporters (Walter and Kissel 1994) have been reported in literature. These studies underline the importance of standardisation of Caco-2 cultures, which would permit comparison of permeability data obtained in different laboratories. The major problem with creating a

standardised culturing procedure between different laboratories is that it is often difficult to put into practice. It would be easier to standardise the experimental conditions, e.g. culturing time and the passage number employed in permeability experiments in different laboratories. Table 5.4 lists the different factors identified that would influence permeability prediction.

In order to better mimic *in vivo* conditions in the small intestine, certain experimental modifications, that are often overlooked in permeability studies, can be applied to the Caco-2 permeability model. The serosal side of small intestine is perfused with blood containing around 4% albumin, which binds to lipophilic compounds providing a necessary driving force for absorption (Krishna *et al.,* 2001). Recently, it was suggested that inclusion of 4% BSA in the basolateral transport medium would minimise the extent of membrane retention of highly lipophilic compounds (Krishna *et al.,* 2001, Saha and Kou 2002, Yamashita *et al.,* 1997). This is because under *in vivo* conditions, retention of lipophilic compounds by the small intestinal membrane is limited due to rapid entry of compounds into the blood circulation. Compounds with high lipophilicity are often bound to serum proteins such as albumin, thereby facilitating their solubility in blood, and enhancing their partitioning out of the enterocytes (Yamashita *et al.,* 1997). Inclusion of 4% BSA in the basolateral wells during permeability experiments demonstrated a 3 to 5-fold increase in the P_{app}s of three highly lipophilic compounds (SCH-A, SCH-B and progesterone) (Krishna *et al.,* 2001). The presence of BSA in the basolateral well reflects *in vivo* sink conditions, thereby resulting in dramatic reduction of cell association. However, this modification to the experimental conditions used in Caco-2 permeability studies has not yet been widely approved. Consequently, Caco-2 permeability experiments were carried out with inclusion of just 0.1% BSA in the present study to maintain integrity of the monolayers. Nevertheless, the P_{app}s of flavonoids were calculated in this study using equation 3 (Section 2.2.3.4), which corrects for membrane retention to determine the effect of membrane retention on the permeability of flavonoids studied. The resulting correlations between cLog Ps and P_{app}s was unchanged compared to the original correlation obtained with equation 2. This indicated that, despite only 0.1% BSA was present in the transport buffer, little membrane retention by the Caco-2 monolayers occurred with the flavonoids during permeability studies. However, previous studies investigating the permeability of flavonoids using the Caco-2 model (Vaidyanathan and Walle 2003, Vaidyanathan and Walle 2001, Walgren *et al.,* 1998, Walgren *et al.,* 2000, Walle *et al.,* 1999) were performed in the absence of BSA in the buffer. To enable comparisons of results obtained in this study with those in the

literature, only 0.1% BSA was included in the buffer to help maintain the integrity of the tight junctions.

A second experimental modification that is also often overlooked is the influence of the unstirred water layer. The UWL is a stagnant layer consists of water, mucus and glycocalyx lining the apical side of the intestinal mucosa, created by incomplete mixing of the luminal contents near the intestinal mucosal surface (Levitt *et al.,* 1988). Agitating the experimental solution has been shown to reduce thickness of the unstirred water layer, thereby minimising its effect on permeability (Avdeef 2001, Naruhashi *et al.,* 2003, Youdim 2003). The thickness of the UWL is usually 1000-1500 μm in the absence of stirring. At a 100 rpm stirring rate, the UWL diminishes to about 300 μm (Adson *et al.,* 1995, Ho 2000). Agitation can be carried out in the form of gas bubbling with O_2/CO_2 (Naruhashi *et al.,* 2003), or by stirring using a rocker (Adson *et al.,* 1995). Experimental attention should also be paid to the buffer pH. Traditionally, the transport buffer used in Caco-2 permeability experiments is buffered at pH 7.4 at both sides of the monolayer. However, under physiological conditions the pH in the upper GI tract ranges from 5.0 to 6.5 (Gary 1996) due to the presence of an acid microclimate just above the epithelial cell layer (Lucas 1983). The pH plays an important role in the ionisation of compounds, and thus on their permeability. To better reflect *in vivo* conditions, different pH values for apical (pH 6.0) and basolateral medium (pH 7.4) were suggested by Yamashita *et al.,* (Yamashita *et al.,* 2000). The authors concluded that P_{app}s obtained under the pH gradient condition showed better correlation with human oral absorption, and are therefore more appropriate for prediction of *in vivo* absorption (Yamashita *et al.,* 2000).

Apart from establishing a common experimental protocol, it is equally important to obtain a standardised culturing condition of Caco-2 cells in order to allow comparison of results obtained from different laboratories. Factors such as the degree of confluence, differentiation of passage, and the variation in choice of media contribute to this problem. On top of this, variations in culturing time following seeding to allow the formation of differentiated monolayers, also contribute to this issue. Owing to the discrepancies in culturing conditions of Caco-2 cells and in the experimental protocols between different laboratories, variable results are often obtained in the P_{app}s of compounds. Differences in the P_{app}s of flavonoids obtained in the present study were seen compared to previous studies (Vaidyanathan and

Walle 2003, Vaidyanathan and Walle 2001, Walgren *et al.,* 1998, Walgren *et al.,* 2000, Walle *et al.,* 1999).

As discussed earlier, it is apparent that the permeabilities of certain flavonoids were not solely dependant upon their lipophilicity. Besides the effects of the aforementioned experimental conditions on compound permeability, the role of efflux pumps in mediating the absorption of drugs, as well as flavonoids, has received much attention (Walgren *et al.*, 1998; Walle *et al.*, 1999; Walgren *et al.*, 2000; Vaidyanathan *et al.*, 2001; Vaidyanathan *et al.*, 2003; Youdim *et al.*, unpublished). The major function of efflux pumps is to confer protection against foreign compounds, by preventing their entry across major barriers in the body. The presence of efflux pumps such as P-gp (Ambudkar *et al.,* 1999, Thiebaut *et al.,* 1987) and multidrug resistance protein 2 (MRP2), an organic anion transporter (Gottesman *et al.,* 2002) has been observed in the intestine where they are localised on the apical surface of epithelial cells. P-glycoprotein transports mainly cationic compounds whereas MRP transports amphipathic anionic conjugates (Paul *et al.,* 1996). Traditionally identification of P-gp substrates using cultured cells can be achieved by comparison of A to B and B to A permeabilities. Results obtained in this study suggest that 3-nitrotyrosine is potentially a substrate of an efflux pump, as its accumulation in cells is limited as a consequence of active efflux. Epicatechin, hesperetin and quercetin but not catechin were proposed to be substrates of efflux pumps, as significantly greater fluxes from the B to A direction were detected compared with that measured from A to B. However, the efflux pump responsible for extruding flavonoids from Caco-2 cells was not identified.

Involvement of the MRP2 in the efflux of flavonoids has previously been reported. Quercetin, epicatechin, epicatechin gallate and chrysin were proposed to be substrates of MRP2 as demonstrated by the increased cellular accumulation in the presence of MK-571, a competitive inhibitor of MRP2 in Caco-2 cells (Vaidyanathan and Walle 2003, Vaidyanathan and Walle 2001, Walgren *et al.,* 1998, Walle 2003, Walle *et al.,* 1999). This result suggests that MRP2 plays an important role in the permeability of the flavonoids studied, and possibly other flavonoids (Vaidyanathan and Walle 2001). In contrast, the only report having described interactions between flavonoids and P-gp, using Caco-2 cells, reported that epicatechin is not a substrate of P-gp (Vaidyanathan and Walle 2001). However, several flavonoids have been reported to interact with P-gp in other cell lines. For instance, the ability of hesperetin, naringenin, quercetin, epicatechin and its O-methylated metabolites to

inhibit P-gp efflux of colchicine in MDCK-MDR1 cells has recently been reported (Youdim *et al.,* unpublished). Other studies have described the inhibitory effect of hesperetin on vincristine efflux from cultured mouse brain endothelial cells (Mitsunaga *et al.,* 2000), and quercetin on P-gp efflux of R123 in rat hepatocytes (Chieli *et al.,* 1995). P-glycoprotein is particularly abundant on the apical surface in enterocytes of the small intestine (Braun *et al.,* 2000, Burton *et al.,* 1993, Hunter *et al.,* 1993), while MRP2 is predominantly expressed in the canalicular membrane of the hepatocytes (Bodo *et al.,* 2003, Fromm *et al.,* 2000). The interaction of flavonoids with P-gp was therefore investigated using MDCK cells transfected with the human MDR1 gene (encoding for P-gp) (Pastan *et al.,* 1988).

The interaction of catechin, epicatechin, hesperetin and quercetin with P-gp in MDCK-MDR1 cells were undertaken using R123, a fluorescent dye that has been widely used as a model substrate compound to study P-gp activity (van der Sandt *et al.,* 2000). Results from this study indicated that catechin was the only flavonoid unable to inhibit efflux of R123 mediated by P-gp at all the concentrations tested. This is in agreement with the lack of B to A transport of catechin. In contrast, epicatechin (100 μM) demonstrated a 10% inhibition of R123 efflux from cultured MDCK-MDR1 cells. The inhibitory effect observed for hesperetin, up to 15% at 100 μM, in the present study is in agreement with Mitsunaga *et al.*, (2000). Amongst all the flavonoids tested in this study quercetin demonstrated the highest inhibition of 40.7 ± 1.8% at 100 μM. The inhibitory effect observed for quercetin was found to be dose-dependent, and in agreement with that reported by Chieli *et al.*, (1995). It was therefore concluded that epicatechin, hesperetin and quercetin were all able to modulate the activity of P-gp in MDCK-MDR1 cells. However, it should be emphasise that none of the flavonoids tested exhibited 50% inhibition on P-gp efflux of R123 at the highest concentration (100μM) tested. Since this concentration is much higher than concentrations likely to be reached *in vivo*, it was concluded that inhibition of P-gp by these flavonoids is unlikely to occur physiologically, apart from in the GI tract where concentration can possibly reach a high level.

The mechanism(s) by which flavonoids modulate P-gp activity are still unclear. The presence of multiple drug binding sites on P-gp could potentially account for the wide range of compounds shown to interact with this transporter (Youdim *et al.*, unpublished). As such, one of the possible mechanisms of P-gp modulation is through direct (specific) interactions, involving either competitive or non-competitive

Absorption and permeability of procyanidin and flavonoids: Caco-2 model

binding of the modulators. P-glycoprotein modulators such as cyclosporin A and verapamil are also substrates of the pump, inhibiting drug transport in a competitive manner (Tsuruo *et al.,* 1981, Twentyman *et al.,* 1990). It was therefore tentatively suggested that epicatechin, hesperetin and quercetin act as inhibitors of P-gp in a similar manner via competitive inhibition of R123 efflux. The absence of inhibitory effects on P-gp mediated efflux of R123 with catechin was possibly due to the fact that it is not a substrate of P-gp. However, it is not certain why catechin is not a substrate of P-gp while epicatechin, its epimer, was demonstrated to be a substrate. As mentioned above, catechin and epicatechin undergo metabolism during passage across the Caco-2 monolayer, suggesting their absorption via the transcellular route in order for them to undergo metabolism. The time a drug resides within the membrane increases the likelihood it will be removed by P-gp. As such, the better absorption of epicatechin than catechin *in vivo* (Baba *et al.,* 2001) may account for this discrepancy. However, no significant difference was found in the P_{app}s of catechin and epicatechin in the current study. Further studies are therefore required to characterise and compare the permeability of these two flavanols.

The work presented in this chapter demonstrated that when the Caco-2 monolayer model was used for predicting the absorption of flavonoids, only a weak correlation exist between lipophilicity of the flavonoids and the observed P_{app}s. Evidence was also obtained for metabolism with some flavonoids such as hesperetin and naringenin. It was reported previously that the Caco-2 model provide permeability ranking of compounds in the same order as more complex *in situ* absorption model (Artursson *et al.,* 2001). In view of this, flavonoids that are of particular importance to the present study, namely catechin, nitrosated catechin and hesperetin as well as 3-nitrotyrosine were further investigated using the isolated small intestine model to characterise the metabolic conversion upon absorption.

CHAPTER SIX

Absorption and permeability of monomeric procyanidins and flavonoids: rat isolated small intestine model

6.1 OBJECTIVES

Previous study investigating the absorption and metabolism of epicatechin has reported the presence of glucuronidated-, 3´-and 4´-*O*-methylated- as well as 3´-*O*-methylated-glucuronide conjugates on the serosal side of enterocytes after perfusion of rat small intestine with epicatechin (Kuhnle *et al.,* 2000). The work presented in the previous chapter demonstrated that nitrosated catechins were not permeable across the small intestine using the Caco-2 model. The purpose of the research described in this chapter was to investigate the extent to which the NCs, potentially formed from catechin and nitrite under the acidic conditions of the stomach, are able to be absorbed via the small intestine. In order to do this:

1) The viability of tissue on the *ex vivo* isolated small intestine model was investigated for studying the absorption of glucose.

2) The *ex vivo* isolated small intestine model for studying the absorption and metabolism of flavonoid compounds was validated by studying the uptake and metabolism of catechin.

3) The ability of the *ex vivo* isolated small intestine model to carry out phase II metabolism, in particular glucuronidation, was established by studying the uptake and metabolism of hesperetin.

4) The absorption and metabolism of NCs using the *ex vivo* isolated rat small intestine model were investigated.

6.2 RESULTS

6.2.1 Absorption of glucose as a marker of jejunal viability

Absorption of glucose was studied initially as a marker of jejunal viability during the course of the absorption study. Glucose absorption across the jejunum is mediated by a family of sodium dependent glucose co-transporters (SGLTs) and by facilitative glucose transporters (GLUTs). Since this process requires energy, the viability of tissue can be assessed by comparing the concentration of glucose in the perfusion buffer and the serosal fluid. The amount of glucose in the serosal fluid was determined at 40 and 80 min perfusion, and the concentration present was calculated using the glucose oxidase colourimetric assay (see Section 2.2.4.2 for details). Glucose (28 mM) was present in the perfusion buffer whereas 51.8 ± 1.6 and 51.5 ± 1.9 mM (n = 3) glucose was present in the serosal fluid after 40 and 80 min perfusion, respectively. Since the concentration of glucose in the serosal fluid after 40 and 80 min perfusion was almost double that initially present in the perfusion buffer, the jejunum was concluded to be viable throughout the experiment.

6.2.2 Absorption and metabolism of hesperetin

Chapter 5 reports that hesperetin exhibited high permeability across the Caco-2 monolayer. It was also found to undergo considerable phase II metabolism resulting in the formation of glucuronidated conjugates. With this in mind hesperetin was studied as a model substrate to compare permeabilities measured using the *in situ* model with the *in vitro* Caco-2 model. In addition, the ability of the isolated rat small intestine to carry out phase II metabolism during perfusion was assessed using hesperetin.

Serosal samples were collected at 20 min intervals, and were analysed by reverse phase HPLC for the presence of hesperetin and its metabolites. The HPLC chromatogram of the perfusion buffer containing 50 μM hesperetin is shown in Fig 6.1A. Didymin (member of the flavanone family) was used as an external standard for this series of analysis. The retention times for didymin and hesperetin were approximately 48.1 and 53.1 min, respectively.

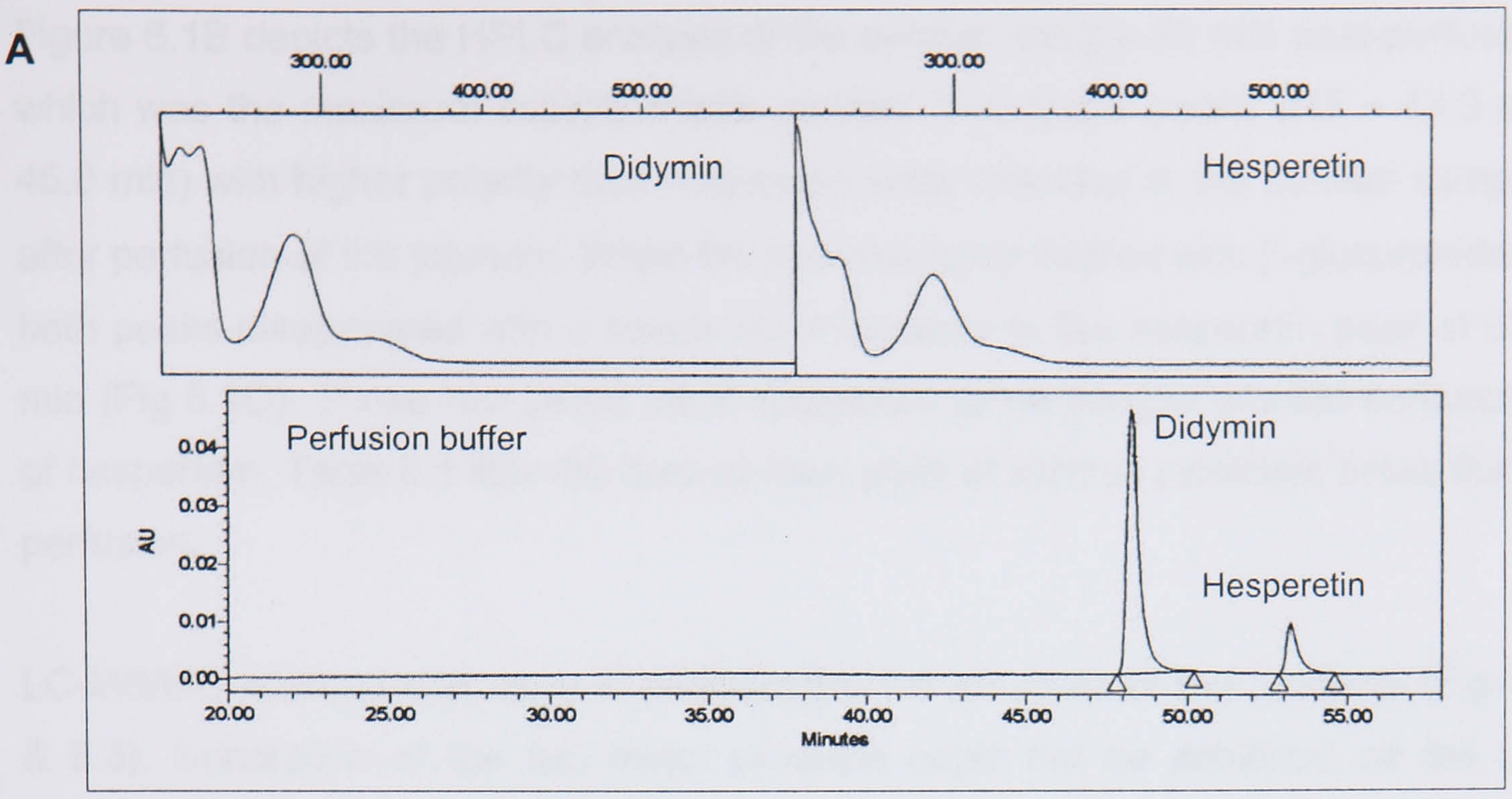


Fig 6.1 50 μM hesperetin perfusion. HPLC analysis of (A) perfusion buffer; (B) serosal fluid 80 min post perfusion; (C) serosal fluid 80 min post perfusion with β-glucuronidase treatment.

Figure 6.1B depicts the HPLC analysis of the serosal sample 80 min post-perfusion, which was the maximum collection time studied. Two major peaks (RT ~ 43.3 and 45.0 min) with higher polarity than hesperetin were detected in the serosal samples after perfusion of the jejunum. When the samples were treated with β-glucuronidase, both peaks disappeared with a concomitant increase in the hesperetin peak at 53.1 min (Fig 6.1C). These two peaks were suggested to be the glucuronide conjugates of hesperetin. Table 6.1 lists the area of each peak at various collection times during perfusion.

LC-MS/MS analysis was used to characterise the structure of the products (Fig 6.2 & 6.3). Separation of the two major products could not be achieved on the LC-MS/MS system, as they coeluted with a RT of 35.8 min. Nevertheless this peak produced a strong signal for *m/z* 479 [hesperetin (MW 302) + glucuronide (MW 176) + H^+] and consequently, both major products were confirmed to be the glucuronide conjugates of hesperetin.

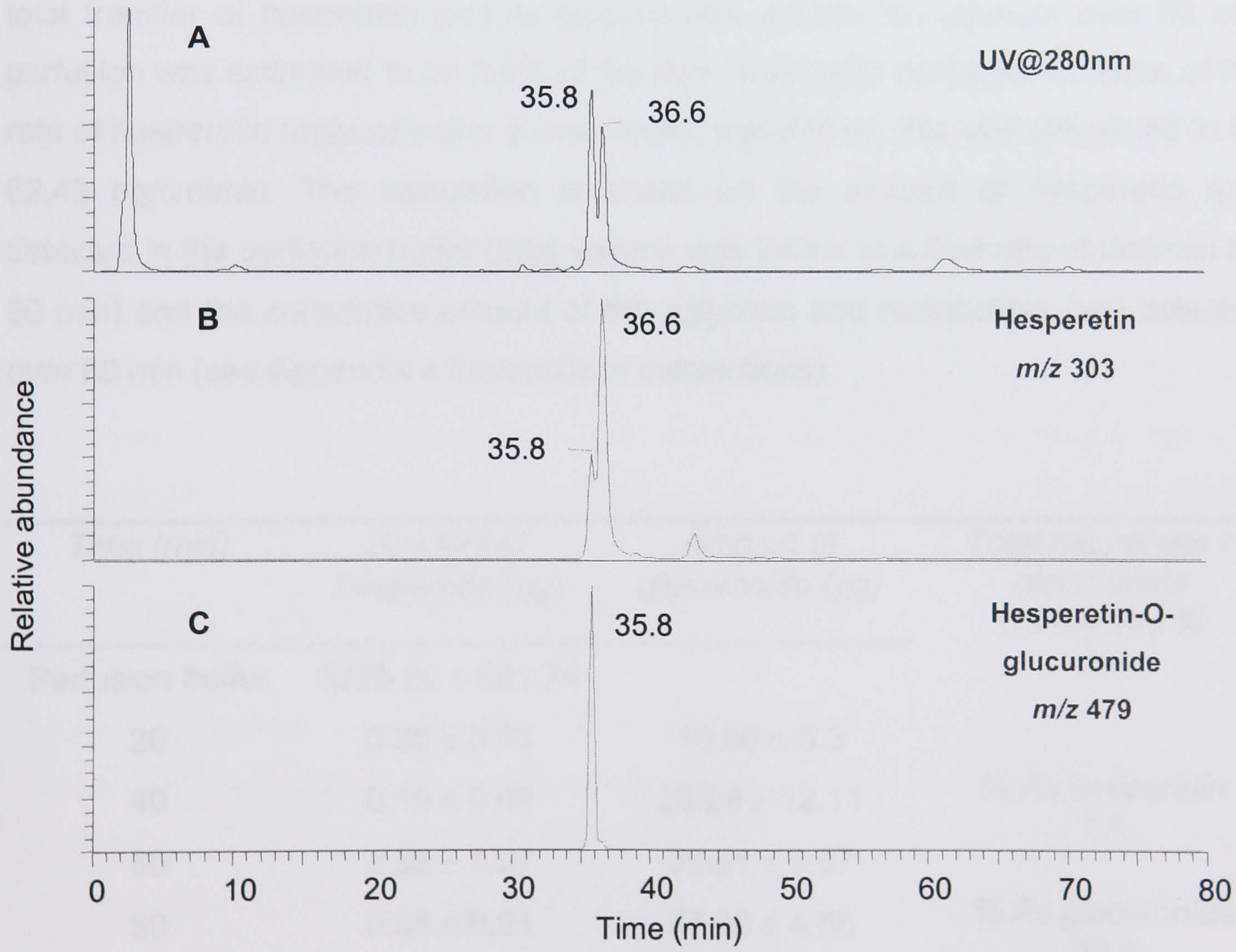


Fig 6.2 Mass spectrometric analysis of samples from hesperetin perfusion. (A) LC-MS/MS chromatograms of the serosal fluid from 50 μM perfusion of hesperetin; (B) PDA-signal (280 nm) of hesperetin; (C) hesperetin-O-glucuronide.

Absorption and permeability of procyanidin and flavonoids: rat isolated small intestine model

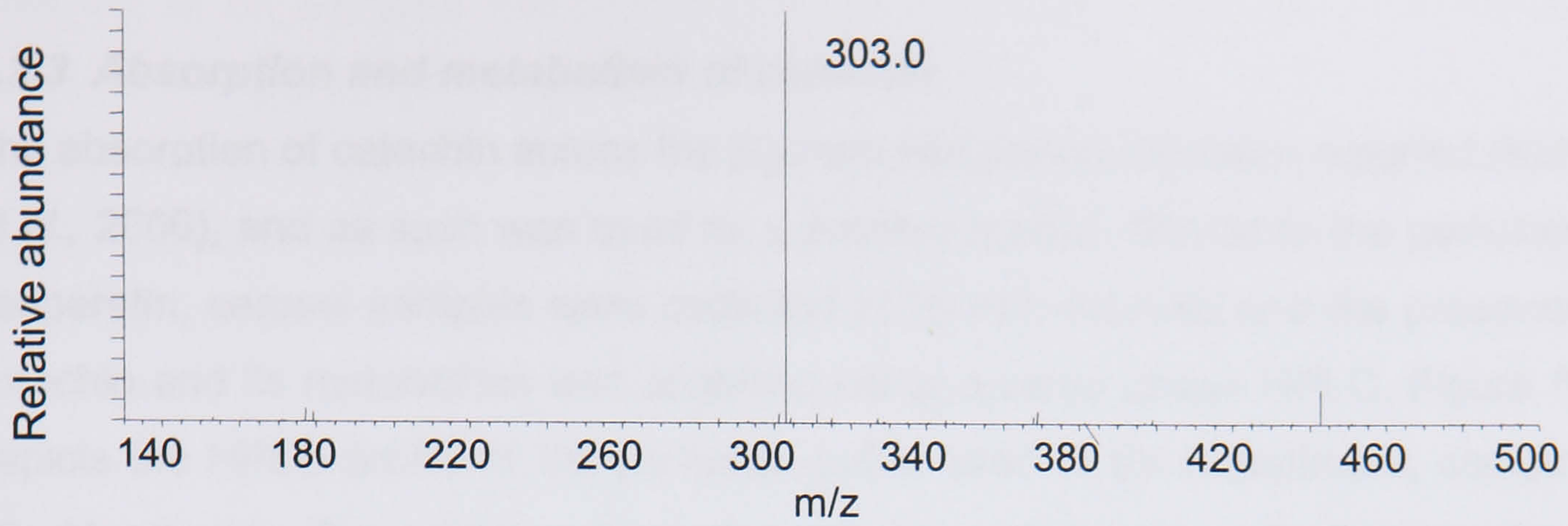


Fig 6.3 Product ion spectra of hesperetin-O-glucuronide (peaks 1 and 2). The major peak is the $[M+H^+]^+$ ion of hesperetin aglycone.

To determine the amount of hesperetin glucuronide present in the serosal fluid, samples were treated with β-glucuronidase. Using a standard curve for hesperetin, the difference in flavonoid aglycone content before and after enzyme treatment was used to calculate the amount of glucuronide conjugates present in the samples. The total transfer of hesperetin and its glucuronides across the jejunum over 80 min perfusion was estimated to be 3.6% of the total hesperetin perfused. In terms of the rate of hesperetin (aglycone and glucuronides) transferred, this was calculated to be 62.48 ng/cm/min. The calculation is based on the amount of hesperetin (μg) detected in the perfusion buffer (total volume was 240ml at a flow rate of 2ml/min for 80 min) and the cumulative amount of the aglycone and metabolites (μg) detected over 80 min (see Appendix 4 for details of calculations).

Time (min)	*Amount of hesperetin (μg)*	*Amount of glucuronide (μg)*	*Total hesperetin + glucuronide transferred %*
Perfusion buffer	3226.89 ± 501.74	-	3.6
20	0.50 ± 0.33	10.66 ± 5.3	
40	0.19 ± 0.09	28.99 ± 12.11	*% As hesperetin*
60	0.35 ± 0.07	35.81 ± 9.07	1.4
80	0.56 ± 0.01	37.89 ± 4.88	*% As glucuronide*
Total absorbed	1.61± 0.55	113.36 ± 33.59	98.6

Table 6.1 Amount of hesperetin and hesperetin glucuronide present in the serosal samples at various collection times. Results are presented as means ± SEM of 6 independent experiments.

Absorption and permeability of procyanidin and flavonoids: rat isolated small intestine model

6.2.3 Absorption and metabolism of catechin

The absorption of catechin across the jejunum has previously been reported (Kuhnle *et al.*, 2000), and as such was used as a positive control. Similar to the perfusion of hesperetin, serosal samples were collected at 20 min intervals and the presence of catechin and its metabolites was analysed using reverse phase HPLC. Figure 6.4A depicts the HPLC profile of the perfusion buffer used in the experiment, containing 50 μM catechin. The retention times for catechin and 4-hydroxy,3-nitrophenylacetic acid (NHPA as external standard) were approximately 32.5 and 46.8 min, respectively.

Figure 6.4B illustrates the HPLC analysis of the serosal fluid 80 min post-perfusion. Three major peaks were detected by HPLC in the serosal fluid, with the largest peak having a similar retention time (~32.0 min) to catechin. However, the UV spectrum for this peak was found to be that for the amino acid tryptophan (Fig 6.4B), as such the presence of catechin was not detected in the serosal sample. The area of each peak at each collection time is shown in Table 6.2. The absence of the native catechin peak in any of the samples collected was probably due to co-elution with tryptophan. Several adjustments of the HPLC gradient were made to test for better separation of catechin and tryptophan (as described in section 2.2.4.3). Adjustment of the HPLC gradient resulted in better separation of catechin from tryptophan, however this caused the co-elution of tryptophan with peak 1. Further attempts using solid phase extraction (SPE) to remove tryptophan from the samples were performed. Although tryptophan is water-soluble, the bulk of it eluted with the methanol fraction, which is also the fraction containing catechin. As a result, the removal of tryptophan by SPE was concluded to be unsatisfactory. Consequently, all samples were analysed using the original HPLC method.

Besides tryptophan, two other new peaks were present in the serosal sample after 80 min perfusion with catechin (Fig 6.4B). Peaks 1 and 2 were suggested to be metabolites of catechin because of the similarity of their spectral characteristics to catechin. They were tentatively identified as glucuronide conjugates as both peaks disappeared following β-glucuronidase treatment (Fig 6.4C). Since catechin co-eluted with tryptophan, no catechin peak was observed after enzyme treatment. However, an increase in the peak area of tryptophan was measured in all samples (Table 6.2), suggesting the formation of catechin following treatment with β-glucuronidase. Moreover the formation of a new peak (peak 3, RT ~ 38.1 min) was

detected in all samples following incubation with β-glucuronidase. Observations made following perfusion with epicatechin (an epimers of catechin) have shown the formation of both glucuronide and O-methyl glucuronide conjugates (Kuhnle *et al.*, 2000). Based upon these findings and the effect of β-glucuronidase treatment observed in the current study, peak 1 was suggested to be the glucuronide conjugate and peak 2 the O-methyl-glucuronide conjugate (according to their sequence of elution), whilst peak 3 was proposed to be the O-methyl conjugate of catechin.

Time (min)	*Catechin[1] (μM)*	*Peak 1*	*Tryptophan[2]*	*Peak 2*	*Peak 3*
Before β-glucuronidase treatment					
Buffer	41.9	-	-	-	-
20	-	9616	434698	5281	-
40	-	49517	411653	27076	-
60	-	104493	516823	55450	-
80	-	130446	522956	65532	-
After β-glucuronidase treatment					
20	-	-	444484	-	10408
40	-	-	413696	-	21289
60	-	-	588898	-	52459
80	-	-	584628	-	57293

Table 6.2 Amount of catechin and metabolites detected at various collection times by HPLC analysis from a representative experiment. [1]Apart from catechin which was shown in concentration (μM), results for all other peaks were presented as peak area due to the lack of available standard for quantification. [2]Tryptophan was presented as peak area for ease of comparison with the other products.

LC-MS/MS was subsequently applied to help identify peaks 1-3. However, all peaks remain unidentified due to their concentration falling below the detection limit. Nevertheless the total transfer of catechin metabolites across the jejunum in 80 min was estimated to be 0.36% of the amount perfused, calculated as previously described in Section 6.2.2.

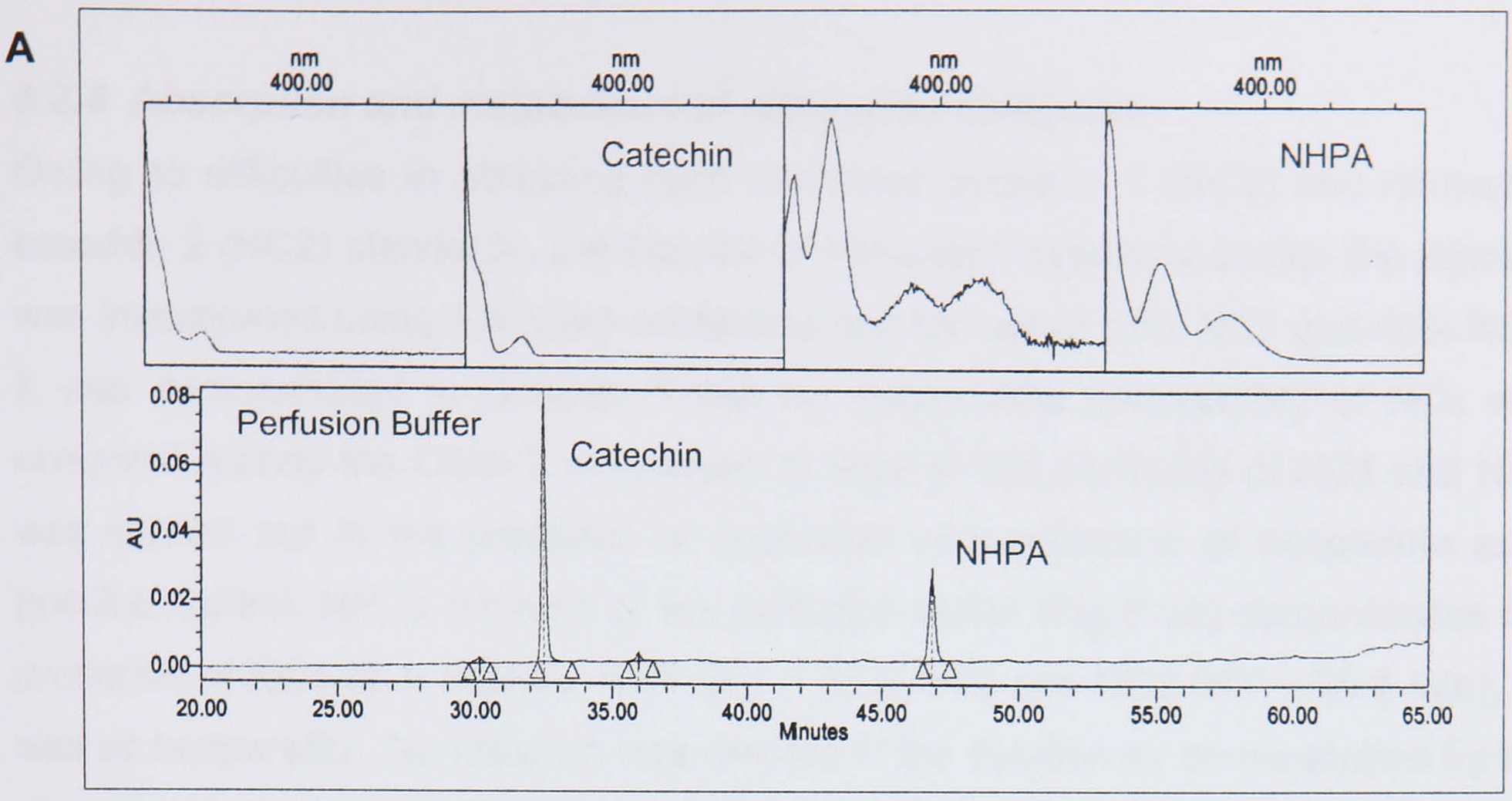
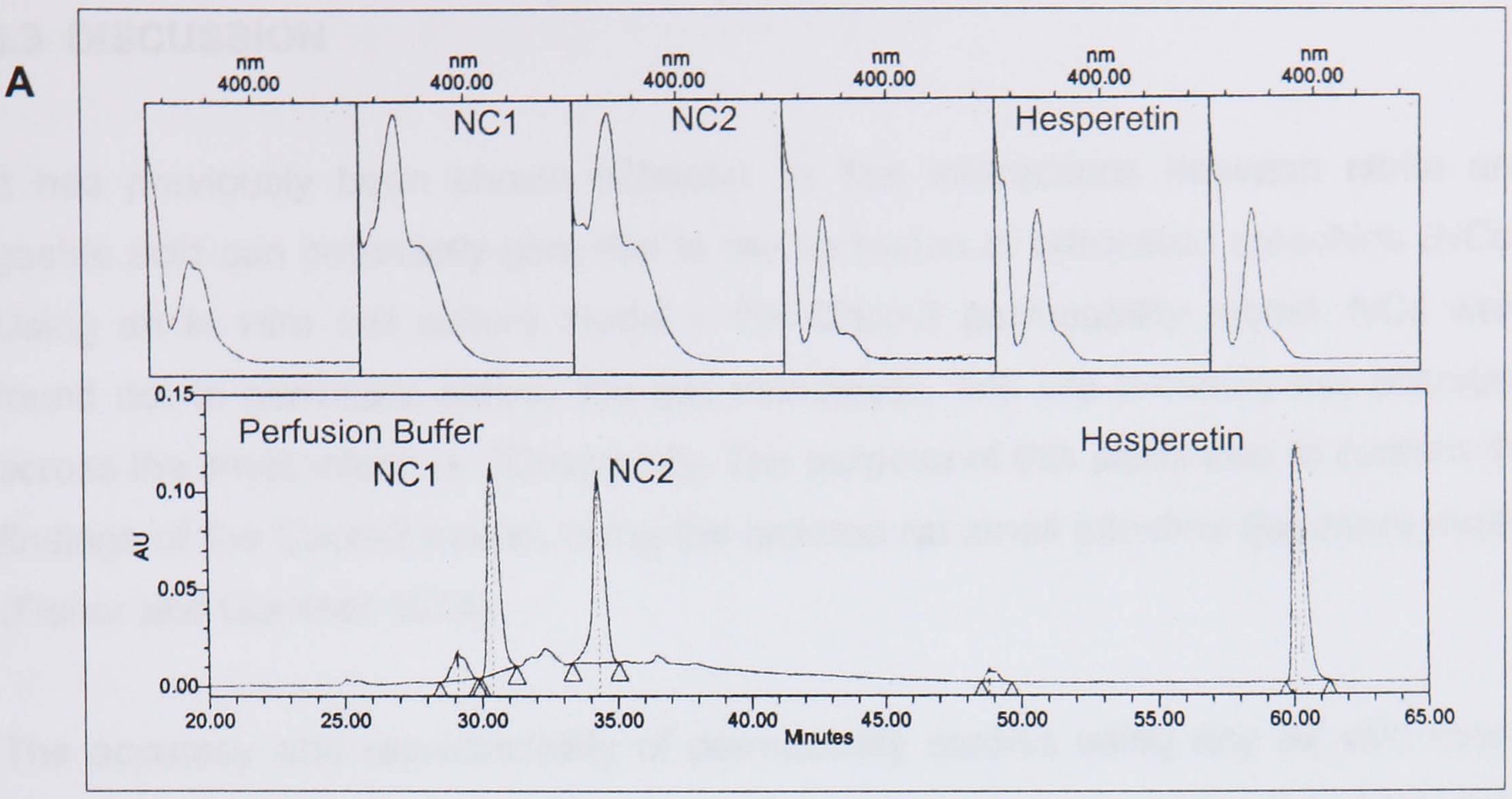


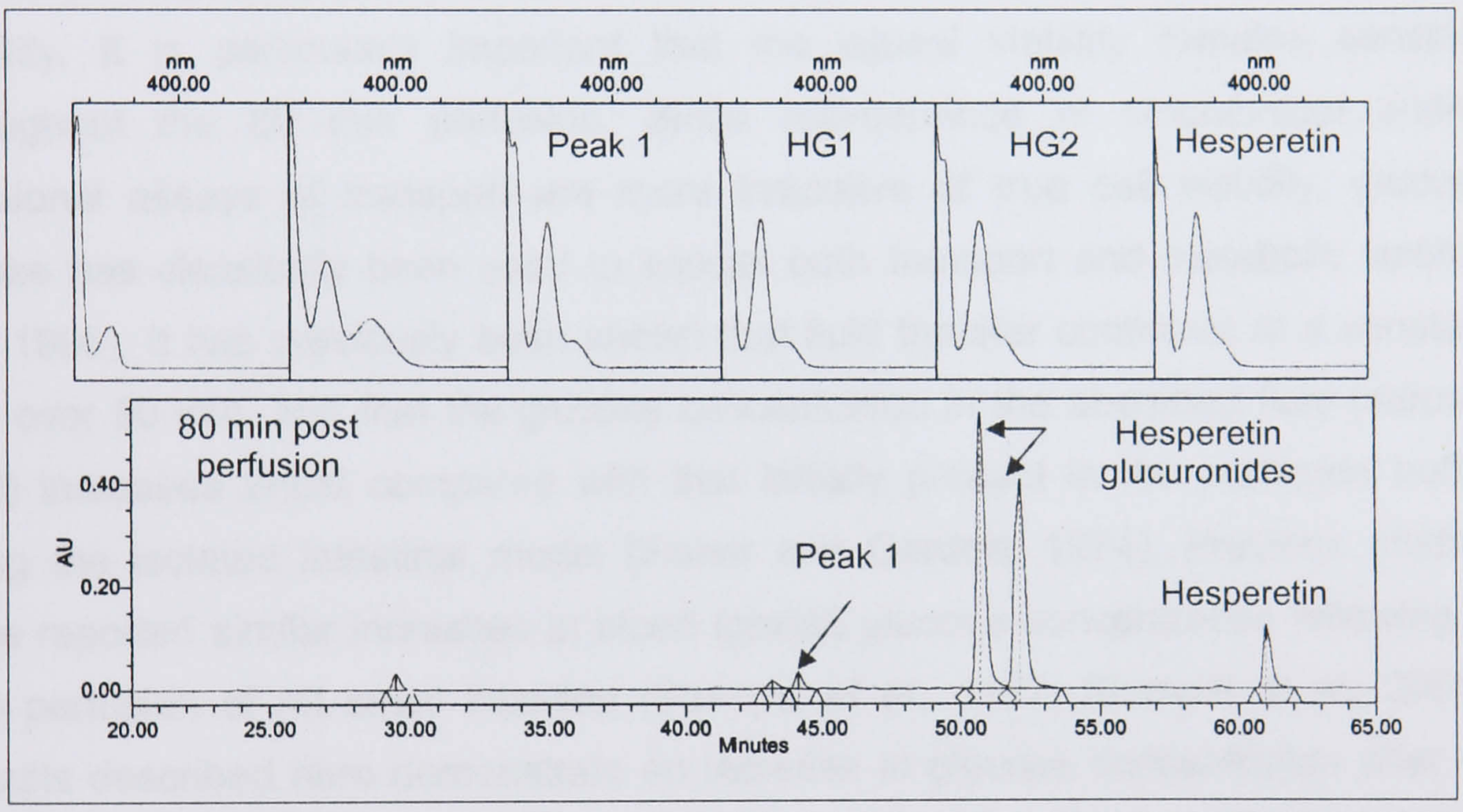
Fig 6.4 50 μM catechin perfusion. HPLC analysis of (A) perfusion buffer; (B) serosal fluid 80 min post perfusion; (C) serosal fluid 80 min post perfusion with β-glucuronidase treatment.

6.2.4 Absorption and metabolism of nitrosated catechins

Owing to difficulties in obtaining pure nitrosated catechin 1 (NC1) and nitrosated catechin 2 (NC2) standards, the transfer of nitrosated catechins across the jejunum was investigated using a mixture containing approximately 52% NC1 and 48% NC2. It was demonstrated in Chapter 5 that no measurable permeability of NCs was observed across the Caco-2 monolayer. In view of this perfusion of NC1 and NC2 was carried out in the presence of equimolar concentrations of hesperetin as a positive control. HPLC analysis of the perfusion buffer (Fig 6.5A) demonstrates the presence of NCs as a mixture: NC1 (RT ~ 31.6 min) and NC2 (RT ~ 35.4 min), as well as hesperetin. No catechin was present in the solution as demonstrated by the absence of a peak at the expected catechins retention time (32.5 min) (Fig 6.5A).

Figure 6.5B shows the presence of both hesperetin and its glucuronide conjugates in the serosal fluid at 80 min perfusion, consistent with that shown in Section 6.2.2. However, no permeability of NCs was observed post perfusion, as no peaks at their expected retention times were observed in the serosal fluid. The serosal sample did not contain any peaks displaying catechin-like spectrum, suggesting no metabolites of nitrosated catechins were present. Consequently, it was concluded that, in contrast to catechin which demonstrated a 0.36% transfer across the jejunum, NCs were not absorbed by the jejunum either as the intact compounds or as their metabolites.





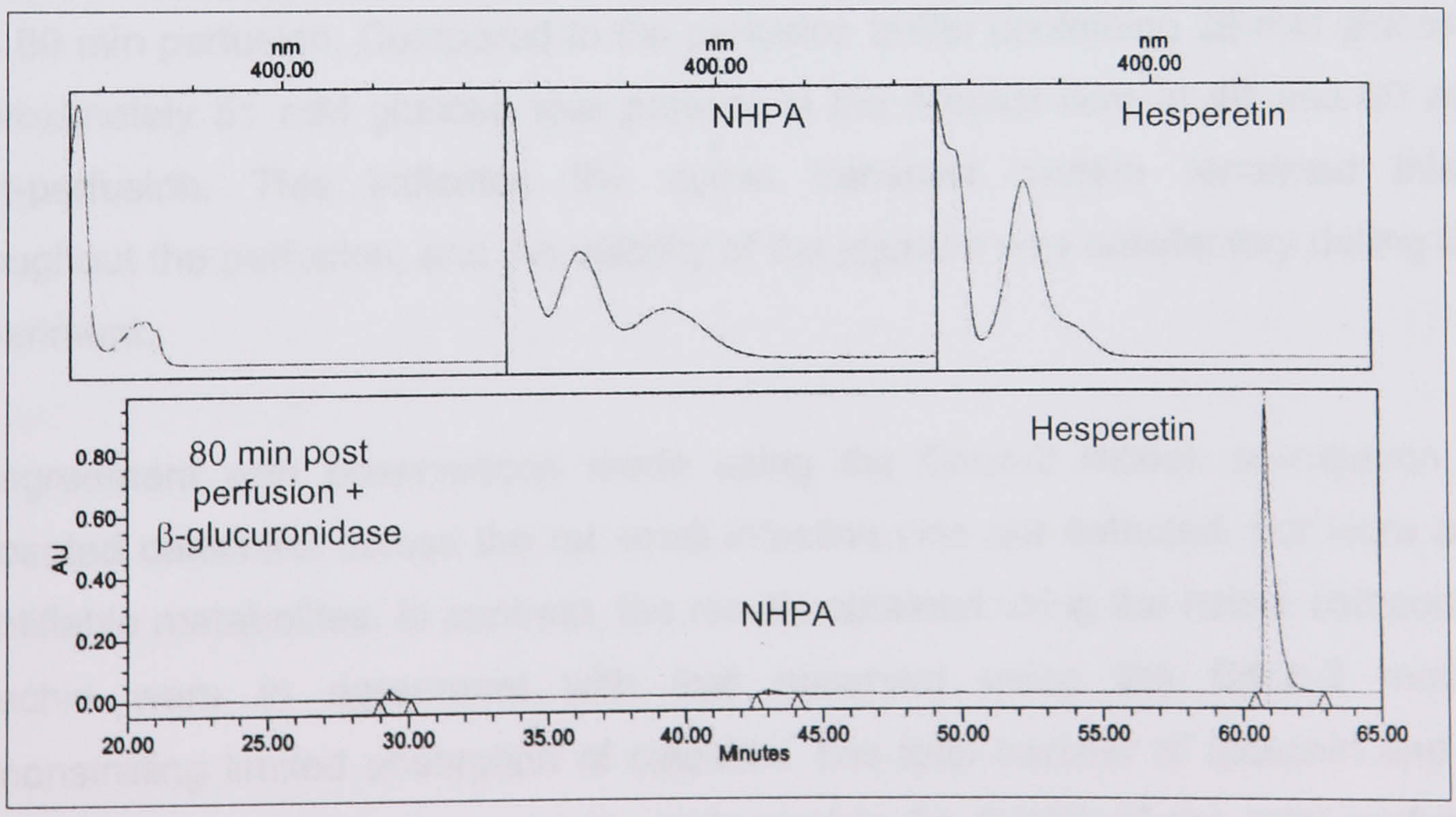


Fig 6.5 50 μM perfusion with NC mixtures. HPLC analysis of (A) perfusion buffer; (B) serosal fluid 80 min post perfusion; (C) serosal fluid 80 min post perfusion with β-glucuronidase treatment. Hesperetin was used as a positive control.

6.3 DISCUSSION

It has previously been shown (Chapter 3) that interactions between nitrite and gastric acid can potentially give rise to the formation of nitrosated catechins (NCs). Using an *in vitro* cell culture model – the Caco-2 permeability model, NCs were found not to permeate across the cell monolayer, and are probably not absorbed across the small intestine (Chapter 5). The purpose of this study was to confirm the findings of the Caco-2 model, using the isolated rat small intestine (jejunum) model (Fisher and Gardner 1974).

The accuracy and reproducibility of permeability studies using any *ex vivo* model rely on utilising tissue that displays normal morphological, biochemical and transport viability. It is particularly important that the jejunal viability remains constant throughout the 80 min perfusion. Since maintenance of biochemical and/or functional assays of transport are more indicative of true cell viability, glucose uptake has classically been used to assess both transport and metabolic viability (Fix 1996). It has previously been shown that fluid transfer continues at a constant rate over 90 min, and that the glucose concentration in the absorbed fluid (serosal fluid) increases 2-fold compared with that initially present in the perfusion buffer using the isolated intestinal model (Fisher and Gardner 1974). Previous studies have reported similar increases in blood (portal) glucose concentration following *in vivo* perfusion of rat small intestine (Stumpel *et al.,* 1997, Stumpel *et al.,* 2001). Results described here demonstrate an increase in glucose concentration after 40 and 80 min perfusion. Compared to the perfusion buffer containing 28 mM glucose, approximately 51 mM glucose was present in the serosal fluid at 40 and 80 min post-perfusion. This indicates the active transport system remained intact throughout the perfusion, and the viability of the jejunum was satisfactory during the experiment.

In agreement with observations made using the Caco-2 model, permeation of nitrosated catechins across the rat small intestine was not detected, nor were any identifiable metabolites. In contrast, the results obtained using the native compound catechin were in agreement with that observed using the Caco-2 model, demonstrating limited absorption of catechin. The total transfer of catechin and its metabolites across the jejunum was estimated to be 0.36% of the total perfused catechin. This figure is extremely low compared to the reported 13.1% absorption of catechin and metabolites through the jejunum in literature (Kuhnle *et al.,* 2000).

Moreover, Kuhnle *et al.*, (2000) reported the presence of a small proportion of catechin aglycones in the serosal fluid, approximately 5% of the total catechin transferred. In contrast, no unmetabolised catechin was found to be absorbed in the present study. The discrepancy between the percentage catechin absorption reported here and that by Kuhnle *et al.*, (2000) was due to differences in the calculations used to estimate absorption. The method used by Kuhnle *et al.*, (2000) is based on the assumption that the perfusion buffer remained static throughout the experiment. The volume of the buffer present was assumed to be equivalent to the volume of the jejunal segment, which was estimated to be 5 ml (i.e. r = 0.25 cm; l = 25 cm). Hence, with the concentration of perfused catechin being 50 μM, the resulting amount of catechin present within any 5ml throughout the duration of the experiment was estimated to be 500 nmol. However, the method here for calculating the rate of perfusion used in the current series of experiments took into account that the isolated small intestine model involved constant perfusion of buffer containing the compound of interest. In this case the flow rate was 2 ml/min for 80 min, therefore the total volume of perfusion buffer the jejunum was exposed to over 80 min was 160 ml, but not 5 ml. Using this more accurate method of calculation, the percentage transfer of catechin across the jejunum over 80 min was found to be 0.36% of the total amount perfused. If the calculation used by Kuhnle *et al.*, (2000) was applied to the results obtained here, the total transfer of catechin would be 17.1%. It is however incorrect to assume the perfused buffer remains static throughout the experiment, and as a result the calculation used by Kuhnle *et al.*, (2000) was less accurate.

It has been widely demonstrated in the literature that flavanols undergo glucuronidation, methylation and sulphation in human and rats upon absorption (Baba *et al.,* 2000, Baba *et al.,* 2001, Baba *et al.,* 2001, Harada *et al.,* 1999, Manach *et al.,* 1999, Piskula and Terao 1998, Spencer *et al.,* 2001, Wang *et al.,* 2000). Although LC-MS/MS failed to determine the identity of the metabolites in the present study (owing to the low concentration present), the pattern of absorption was similar to the literature. Glucuronide conjugates with higher polarity than catechin were identified by β-glucuronidase treatment. These two metabolites were tentatively suggested to be the O-methyl-glucuronide and O-methyl conjugate, due to the disappearance of the former following β-glucuronidase treatment and a corresponding increase in the latter. The order of elution of our metabolites and their suggested identity are in agreement to that reported by Kuhnle *et al.*, (Kuhnle *et al.,*

2000); glucuronide, aglycone, O-methyl-glucuronide and O-methyl conjugate. In addition, no unmodified flavanols were found to be absorbed, which is consistent with the literature showing little or no absorption of the parent compounds (Donovan *et al.,* 2001, Kuhnle *et al.,* 2000).

To evaluate the potential of the isolated rat small intestine model to carry out phase II metabolism, such as glucuronidation, the permeability of hesperetin was studied. A much higher total transfer of 3.6% of hesperetin compared to catechin (0.36%) was demonstrated by perfusion of 50 μM hesperetin. The majority (98.6%) of the transferred hesperetin was found to be the glucuronide conjugates, demonstrating the ability of the isolated jejunum to carry out phase II metabolism. The results obtained here is in agreement with the Caco-2 experiments (Section 5.2.3), describing high permeability of hesperetin and the formation of glucuronidated metabolites. In contrast, in a similar study performed by Spencer *et al.*, (Spencer *et al.,* 1999), high levels (82.5%) of hesperetin aglycone were detected in the serosal fluid. It is now widely accepted that flavonoids are absorbed and detected mainly as metabolites of glucuronides, methyl and sulphate conjugates (Rice-Evans *et al.,* 2000). Indeed, evidence to support the results described here can be found in a recent feeding study of hesperedin, the naturally occurring glycosidic form of hesperetin, in which 87% of the circulating forms of hesperetin was found to be the glucuronides (Manach *et al.,* 2003).

In Chapter 5 the permeabilities of 16 flavonoids were calculated using the Caco-2 permeability model, and the relationship between permeability and lipophilicity was determined. A weak correlation was obtained, suggesting the lipophilicity/polarity of compounds is only one of many factors affecting their permeability. Another possibility for the greater permeability observed with hesperetin compared to catechin and nitrosated catechins is differences in their susceptibility to undergo oxidation. The oxidisibility of hydroxycinnamates has been suggested previously to influence the extent of absorption in the jejunum (Spencer *et al.,* 1999). For instance, ferulic acid was absorbed (6.69 nmol/20 cm gut/5 μmol perfused) to a much greater extent than the catechol-containing monophenolics caffeic acid (0.69 nmol/20 cm gut/5 μmol perfused) or its quinic ester (0.115 nmol/20 cm gut/5 μmol perfused). It is not surprising the same was found to be true for flavonoids in the results described here, with greater absorption seen with the more stable hesperetin compared with the more oxidisable molecule catechin.

In contrast, it is not fully understood why the addition of nitroso groups hindered uptake of the resulting nitroso catechin by the jejunum. An apparent paradox relates to the fact that catechin and hesperetin were mainly absorbed across enterocytes and detected as glucuronide conjugates, which are usually attached to the 5 or 7 position on the A ring of their structures; since nitrosation of catechin was proposed to occur at the 6 or 8 positions on the same ring of catechin, it is possible that addition of the nitroso groups results in steric hindrance, preventing glucuronidation of catechin, and subsequent absorption (Donovan *et al.,* 2001, Kuhnle *et al.,* 2000, Natsume *et al.,* 2003, Spencer *et al.,* 2001). However, this does not explain the factors that hinder the initial uptake of nitrosated catechins, unless there is the possibility that glucuronidation might be a cellular membrane event. Another possibility could have been that interaction of nitrosated catechins with efflux transporters was responsible for their efflux back into the small intestine, and hence their inability to be absorbed by the enterocytes. On the other hand, studies undertaken in Chapter 5 (comparison of apical to basolateral with basolateral and apical P_{app}s) suggested NCs are unlikely to be substrates of efflux pumps, therefore the second proposal seems unlikely.

The mechanism by which hesperetin and catechin are absorbed by the small intestine is not clear. Given the high lipophilicity of hesperetin and the extensive metabolism that must occur within the enterocytes, hesperetin was suggested to be absorbed via the transcellular pathway. A previous study using Caco-2 cells has demonstrated the transport of catechin via the paracellular pathway (Deprez *et al.,* 2001). However, no intact catechin was detected in the serosal fluid in this study, with all of the absorbed catechin being in conjugated forms. Catechin must have entered the cells to have undergone metabolism, and hence, paracellular transport is probably not the mechanism of uptake in the rat jejunum. Recently, it has been shown that quercetin glucosides are transported by the glucose carrier SGLT-1 across the brush border membrane of rat small intestine (Ader *et al.,* 2001, Day *et al.,* 2003, Walgren *et al.,* 2000). Since the glucose moiety is crucial to the ability to interact with the SGLT-1, quercetin aglycone was not able to interact with the transporter (Ader *et al.,* 2001). Little information is available in the literature with regard to the interaction of flavonoids and possible uptake by other transporters. A rapid, energy-dependent transport system was suggested to be present in mammalian aortic endothelial cells for the uptake of morin, as shown by the several-fold increase in morin uptake when ATP was added to the culture medium

(Schramm *et al.,* 1999). The authors further suggested that the transport system in these cells uses hydroxylated phenolic compounds as substrates. However, it is not clear whether such transporters, or other active transporters capable of transporting flavonoids, are present in enterocytes forming the small intestine. If flavonoids can be transported via this mechanism, the addition of nitroso groups to catechin may influence the way it interacts with the transport system, thereby affecting their uptake into cells. Further studies are required to elucidate the mechanisms involved in the transfer of hesperetin, catechin and nitrosated catechins.

The rat intestinal perfusion model is one of the most popular methods for determining drug absorption and metabolism, and has yielded much information about mechanisms of transport and metabolism of flavonoids (Andlauer *et al.,* 2000, Andlauer *et al.,* 2001, Crespy *et al.,* 2001, Donovan *et al.,* 2001, Gee *et al.,* 2000, Kuhnle *et al.,* 2000, Kuhnle *et al.,* 2000, Spencer *et al.,* 2001). However, most perfusion studies in the literature are carried out *in situ* (Barthe *et al.,* 1999, Bohets *et al.,* 2001, Donovan *et al.,* 2001, Fagerholm *et al.,* 1996, Fagerholm *et al.,* 1999, Kim *et al.,* 1993, Lennernäs 1997, Liu and Hu 2002, Stewart *et al.,* 1995, Zheng *et al.,* 1994) and are different to the current approach involving the use of isolated tissue submerged in paraffin oil. *In situ* perfusion of the small intestine involves cannulating segments of the intestine of anaesthetised animals. A buffer containing the test compound is perfused at a constant flow rate while the animal is conscious. The perfusate is then collected at timed intervals, and analysed for the amount of the test compound present. Absorption is calculated as the difference in the amount perfused and the amount recovered from the effluent after perfusion, assuming that disappearance of the compound from the intestinal lumen accurately reflects absorption. However, permeability will be overestimated if the test compound is extensively metabolised, or if there is significant accumulation of the compound within the intestinal membrane. To overcome this problem sampling from the mesenteric vein (the blood draining from the perfused intestinal segment) and the portal vein for analysis of absorbed compound and metabolites can give a more accurate picture of the overall absorption profile (Barthe *et al.,* 1999, Bohets *et al.,* 2001). The advantage of this model is that it permits determination of metabolism and excretion at other sites of the body, such as the liver. This allows understanding of how different organs function together in the living animal (Donovan *et al.,* 2001), and providing a complete picture of the metabolism of a compound. Permeability data for passively transported compounds obtained from the *in situ* model has been shown to correlate well with the Caco-2 model (Kim *et al.,* 1993, Zheng *et al.,* 1994),

and with human perfusion model (Bohets *et al.,* 2001, Fagerholm *et al.,* 1996, Lennernäs 1998). However, similar data comparing the permeability of compounds in isolated intestinal perfusion and Caco-2 cells is not available. Furthermore, the correlation between permeability estimated in the isolated intestinal model and with human perfusion remains to be established.

The major advantage of the isolated *ex vivo* intestinal model is that it allows absorbed fluid to be collected directly in the same form as if it were transferred to the mesenteric circulation, thus eliminating any complications associated with liver metabolism (Kuhnle *et al.,* 2000). In addition, anaesthetics and surgery may cause anoxia and decrease intestinal blood flow, intestinal motility and energy supply, resulting in decreased passive and active transport (Uhing and Kimura 1995, Yuasa *et al.,* 1993). The choice of anaesthetic and surgery would have a relatively limited effect on permeability estimation in the *ex vivo* model compared to the *in situ* set up.

A modification was suggested for future experiments using the isolated small intestine perfusion model in order to improve accuracy of the results. A marker compound, such as PEG 4000, that is not permeable though the jejunal barrier should be included in future experiments for continuous monitoring of intestinal integrity (Fagerholm *et al.,* 1996).

In conclusion, the work presented here demonstrated that catechin was extensively metabolised upon absorption in the jejunum, and the permeability of catechin was around 0.36% of the total perfused. Hesperetin, a more lipophilic flavonoid showed a higher percentage absorption at 3.6%, with the majority being the glucuronide conjugates. Nitrosated catechins, on the other hand, were not absorbed across the small intestine. Assuming no absorption of nitrosated catechins occurs in the colon or the stomach, they are probably not able to elicit any biological effects on cells beyond the GI tract. As such, the important question arising from this concerns the potential effect of NCs on the gastrointestinal tract with respect to their biological activity and cytotoxicity.

CHAPTER SEVEN

Biological properties of nitrosated catechins

7.1 OBJECTIVES

There is considerable interest in the health benefits of dietary flavonoids due to their abilities to scavenge reactive oxygen and nitrogen species. The results presented in this thesis so far demonstrate that monomeric and dimeric procyanidins inhibit acidic nitrite-mediated tyrosine nitration via a novel mechanism of nitrosation. These nitrosated products derived from catechin were not permeable across the small intestine, studied using the *in vitro* Caco-2 model and an *ex vivo* isolated rat small intestinal model. However, because of the known carcinogenic properties of N-nitrosamines, it is important to determine the biological properties of nitrosated catechins, and in particular their potential cytotoxicity. The present study was designed to address:

1) The potential cytotoxicity of nitrosated catechins (NCs) by studying their effects on the proliferation of Caco-2 cells as a model of the intestine.

2) The ability of NCs to protect cells against oxidative stress-induced damage, (as had been shown for the non-nitrosated compounds) using a human fibroblast model (Spencer *et al.,* 2001). To achieve this, several experiments were designed:

- To establish the toxicity of NCs and catechin in this cell model.
- To identify suitable conditions of oxidative stress, in the form of H_2O_2, leading to 50% cell death.
- To investigate the ability of NCs to protect against oxidative stress-induced damage in the human fibroblast model.

7.2 RESULTS

7.2.1 Toxicity of nitrosated catechins – Caco-2 cells

Chapter 3 reports that catechin can potentially confer protection against RNS generated from acidic nitrite via a mechanism of competitive nitrosation. Two dinitrosated catechins are produced from this interaction, with the proposed position of nitrosation to be on the A ring. Before a conclusion can be made on the health benefits of this interaction, it is important to establish the biological properties of the NCs, relative to native catechins.

Caco-2 cells were originally derived from a human colonic cancer cell line (Fogh 1977), but exhibit characteristics of the small intestine if cultured for 21 days. The potential toxicity of NCs to Caco-2 cells was investigated following their continuous supplementation for 21 days. Caco-2 cells were exposed to nitrosated catechins 24h post-seeding, and their effect on cell proliferation examined over 21 days using the sulforhodamine B (SRB) assay. Sulforhodamine B is a protein stain widely used for the quantification of cellular proteins of cultured cells (Skehan *et al.,* 1990). Total protein mass, which is related to the cell number, can be estimated by colourimetric measurement of the bound dye (SRB binds to basic amino acids of cellular proteins) (Skehan *et al.,* 1990), and calculated from a standard graph generated from known cell numbers (see Appendix 5). The effect of NCs on cell proliferation following exposure for 7 and 14 days was also studied to examine any effects that might have occurred during transformation of Caco-2 cells from a colonic to intestinal phenotype. However, cell number after 14 and 21 days cultured could not be determined using the standard graph because the absorbance (relative to number of cells) fell outside the linear region of the standard graph (see Appendix 5). As such the toxicity of NCs on Caco-2 cells after 7, 14 and 21 days exposure was reported as the absorbance of SRB at 490 nm. Toxicity of the test compounds was evaluated as their potential (long-term) influence on cell proliferation.

Figure 7.1A illustrates the effects of NCs (containing approximately 52% NC1 and 48% NC2) and catechin on the proliferation of Caco-2 cells following 7 days exposure. No significant differences were observed between SRB absorbance at 490nm (relative to cell numbers measured, see Appendix 5) in control wells and those supplemented with

either NC or catechins, at all concentrations studied. This observation suggests that neither nitrosated catechins nor catechin exert toxic effects towards cell proliferation following continuous exposure for 7 days. Although no significant differences were observed between the proliferation of control and treated cells, cell proliferation was significantly lower following exposure to 30 μM catechin as compared with NCs supplementation at 3 and 30 μM ($P<0.05$ in each case). Figure 7.1B illustrates the effects of NCs and catechin on the proliferation of Caco-2 cells following 14 days continuous supplementation. The effects observed were similar to that observed following 7 days exposure, with no significant differences observed between cell numbers measured in control wells and those supplemented with either NC or catechin, at all concentrations studied. Although one-way ANOVA did not show there to be any significant effects on cell proliferation with either NCs or catechins as a function of increasing concentration, post-hoc analysis did identify the effect of 3 μM nitrosated catechins on cell proliferation to differ from that at 10 and 30 μM ($P<0.05$ in each case). No toxic effects, towards cell proliferation were seen following 21 days exposure to NCs and catechins (Fig. 7.1C). A significant increase in SRB absorbance was measured in wells treated with NCs and catechin (at all concentrations studied) compared with the absorbance measured in the control cells ($P<0.05$ in all cases). However, no significant differences were observed between NCs and catechin on cell proliferation, at any concentrations tested.

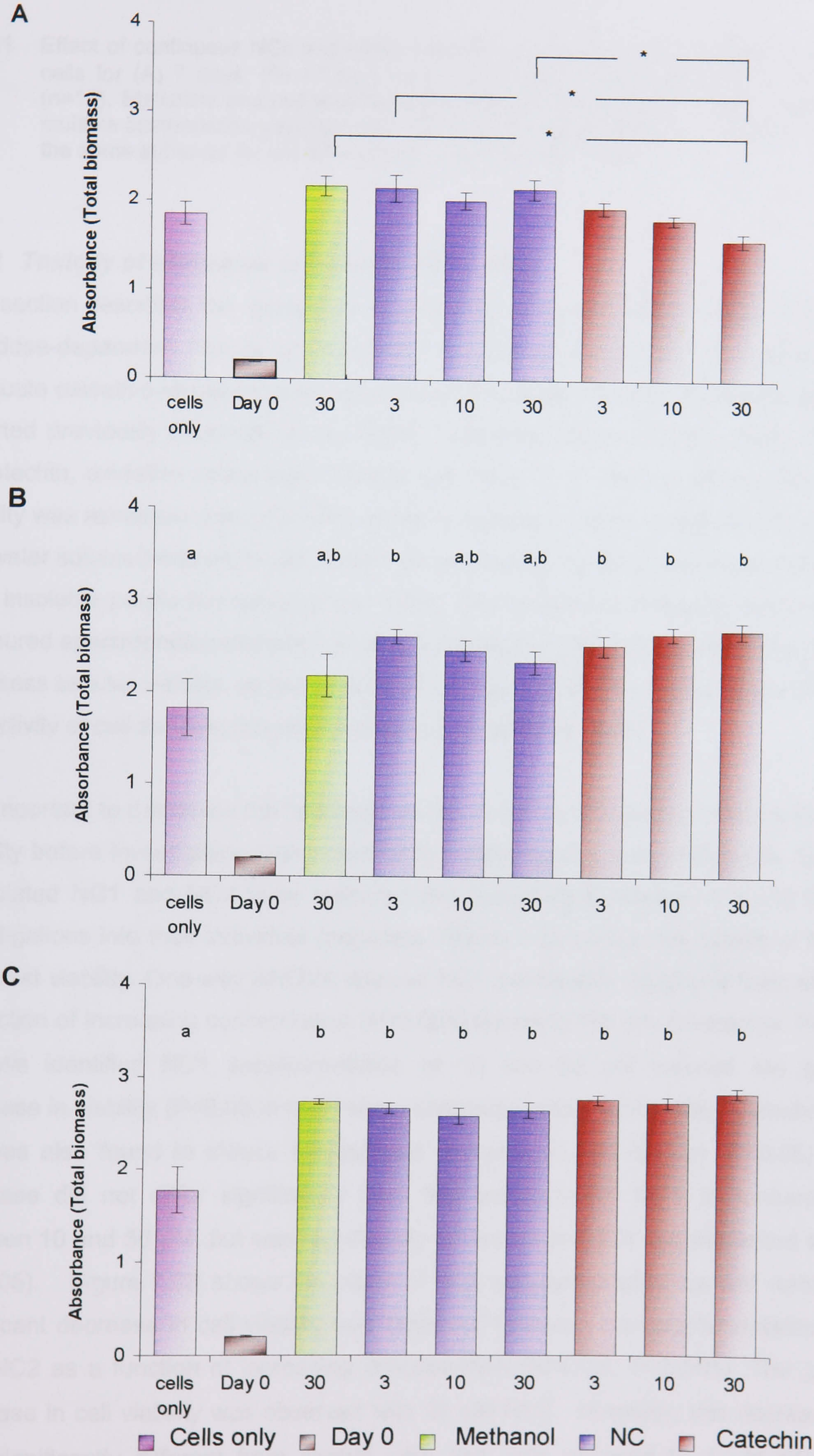

A
B
C
Absorbance (Total biomass)
0
1
2
3
4
cells only
Day 0
30
3
10
30
3
10
30
*
*
*
a
a,b
b
a,b
a,b
b
b
b
a
b
b
b
b
b
b
b
Cells only
Day 0
Methanol
NC
Catechin

Fig 7.1 Effect of continuous NCs and native catechin exposure on the proliferation of Caco-2 cells for (A) 7 days; (B) 14 days and (C) 21 days. Values represent mean ± SEM (n=16). Statistical analysis was performed using one-way ANOVA with Tukey-Kramer multiple comparisons post *hoc* tests (*P<0.05). At days 14 and 21, treatments sharing the same subscript do not differ significantly from each other.

7.2.2 Toxicity of nitrosated catechins - FEK4 cells

This section describes the application of an *in vitro* human fibroblast model to examine the dose-dependent toxicity of nitrosated catechins. The ability of epicatechin to attenuate oxidative-stress-induced cell damage in cultured human fibroblasts has been reported previously (Spencer *et al.,* 2001). Following 18h pre-treatment of cells with epicatechin, oxidative stress was induced with H_2O_2. The effect of epicatechin on cell viability was assessed using the MTT assay to evaluate cellular metabolic activity. MTT is a water soluble tetrazolium salt, which can be cleaved by dehydrogenase enzymes to yield insoluble purple formazan (Slater 1963). The amount of formazan produced was measured spectrophotometrically following solubilisation with DMSO. MTT can be used to assess cellular viability, as the amount of formazan produced is directly correlated to the activity of cellular dehydrogenase enzymes (Mossman 1983).

It is important to determine the 'inherent' toxicity of NCs and catechin towards fibroblast viability before investigating their potential to protect against oxidative stress. Samples of isolated NC1 and NC2 were prepared (as described in Section 2.2.2.5) to allow investigations into their individual properties. Figure 7.2A shows the effects of NC1 on fibroblast viability. One-way ANOVA showed NC1 exhibited a significant toxic effect as a function of increasing concentration (P<0.001) following 18h pre-incubation. Post-hoc analysis identified NC1 supplementation at 10 and 50 μM induced the greatest decrease in viability (P<0.05 in each case) compared with control cells. Catechin at 30 μM was also found to induce a significant decrease in cell viability (P<0.05). This decrease did not differ significantly from that induced with NC1 at concentrations between 10 and 50 μM, but was significantly different from NC1 supplemented at 3 μM (P<0.05). Figure 7.2B shows the effect of NC2 supplementation on cell viability. A significant decrease in cell viability was observed following pre-supplementation (18h) with NC2 as a function of increasing concentration (ANOVA, P<0.001). The greatest decrease in cell viability was observed with 50 μM NC2. However, this decrease was only significantly different from control cells and cells exposed to 3 μM NC2. No

significant differences were observed between the overall effects of NC1 and NC2 (Two-Way ANOVA, P>0.05).

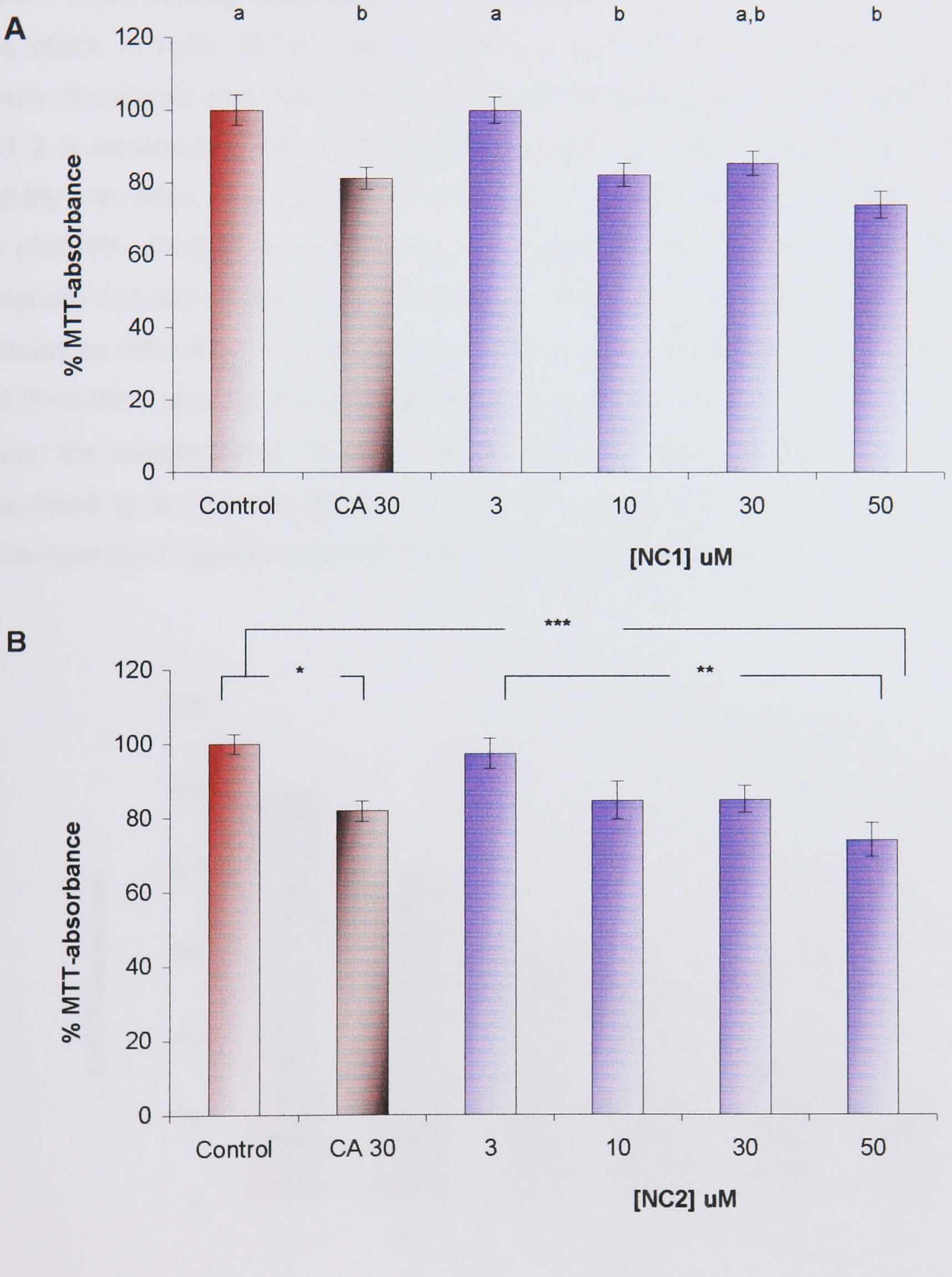


Fig 7.2 Toxicity of NCs and catechin on cellular function in fibroblasts after 18h pre-treatment of test compounds. (A) NC1, (B) NC2. Data are presented as means ± SEM [n=8]. Statistical analysis was carried out using one-way ANOVA with Tukey-Kramer multiple comparisons post *hoc* tests (*P<0.05; **P<0.01, ***P<0.001). In experiments with NC1, treatments sharing the same subscript do not differ significantly from each other.

7.2.3 Protection of nitrosated catechins against peroxide-induced oxidative stress

7.2.3.1 H_2O_2 toxicity towards FEK4 cell viability

The effect of H_2O_2 (0-125 μM) exposure on oxidative stress-induced cell death of human fibroblasts was examined using the MTT assay. Figure 7.3 shows the effect of 1 and 2 h incubations with increasing H_2O_2 concentrations. A significant decrease in viability was seen as a function of increasing H_2O_2 concentrations incubated for 1 and 2h (ANOVA, $P<0.01$ in each case). No significant differences in cell viability were observed between 1 and 2h incubations of H_2O_2 at 25-75 μM. However, significant differences following 1 and 2h exposures were only observed at 100 ($P<0.01$) and 125 μM ($P<0.001$), with 2h exposure inducing greater decrease in viability. In the present study, the concentration found to induce 50% cell death following 2h exposure was calculated to be 75 μM (50.0 ± 4.5%). This concentration and exposure time was subsequently chosen to examine the protective effects of flavonoids.

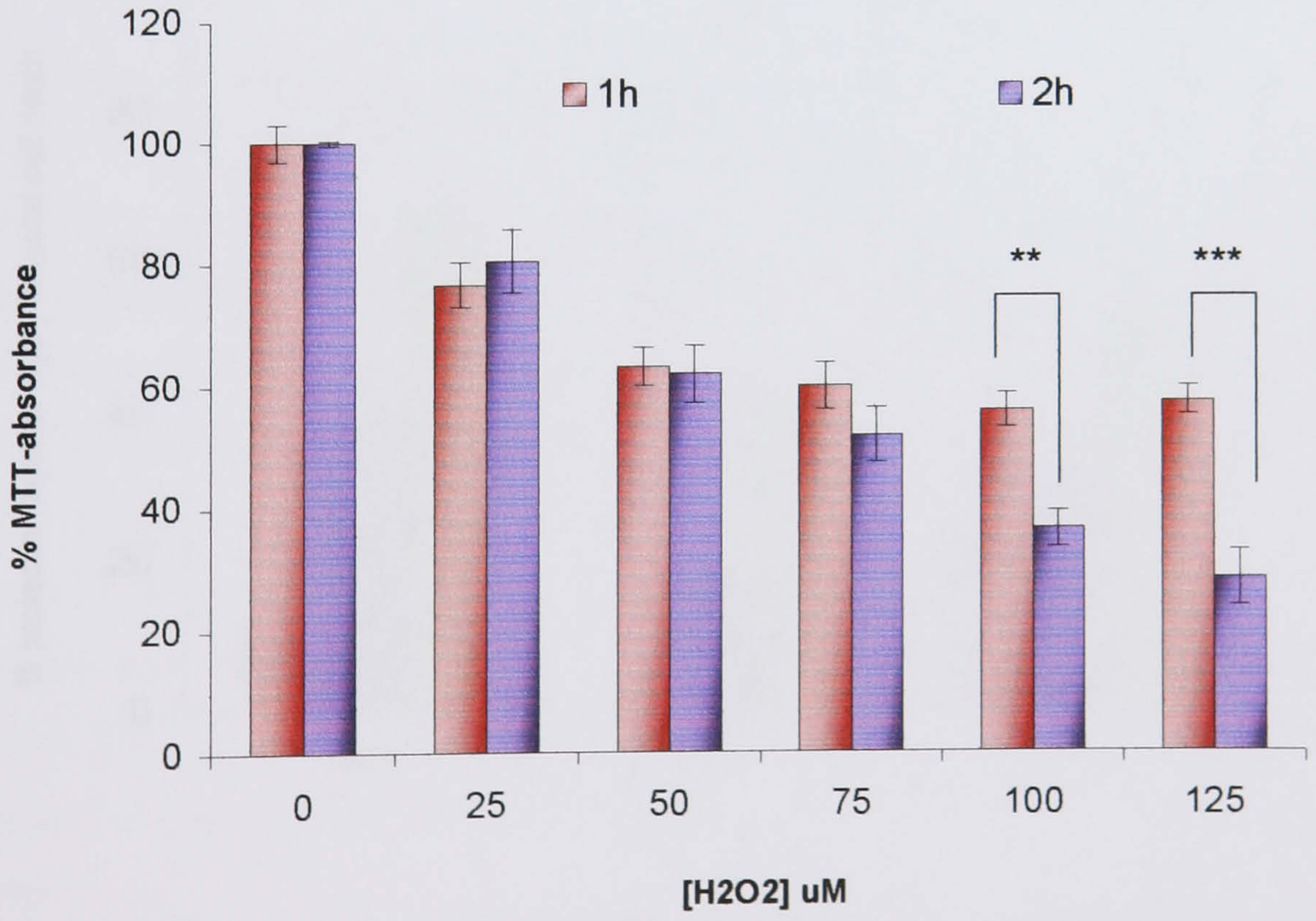


Fig 7.3 The effect of H_2O_2 concentration and exposure time on human fibroblasts viability, as assessed using the MTT assay. Data is presented as means ± SEM. [n=8]. (**P< 0.01; ***P< 0.001, unpaired Student's *t*-test).

7.2.3.2 Protection against H_2O_2-induced cytotoxicity

The ability of nitrosated catechins to protect against H_2O_2-induced (75 μM, 2h) cell death in human fibroblasts was investigated using the MTT assay. The protective effects of NCs and catechin were calculated as a percentage of the increase in cellular activity compared to cells without treatment of NCs or catechin, i.e. cells without treatment offered 0% protection. The results are illustrated in Fig. 7.4. A dose-dependent attenuation in the loss of cell viability was observed with all three compounds (ANOVA, $P<0.001$ in each case). No significant differences were observed between the protective effects of NC1 and those afforded by catechin at the same concentrations. By contrast, the protection afforded by NC2 at 3 μM was significantly lower than catechin at the same concentration ($P<0.05$). No significant difference was observed between NC2 and catechin at 10 or 30 μM. Comparisons between NC1 and NC2 found significantly greater protection afforded by NC1 at 10 and 30 μM ($P<0.01$ and 0.001 respectively), but not at 3 μM.

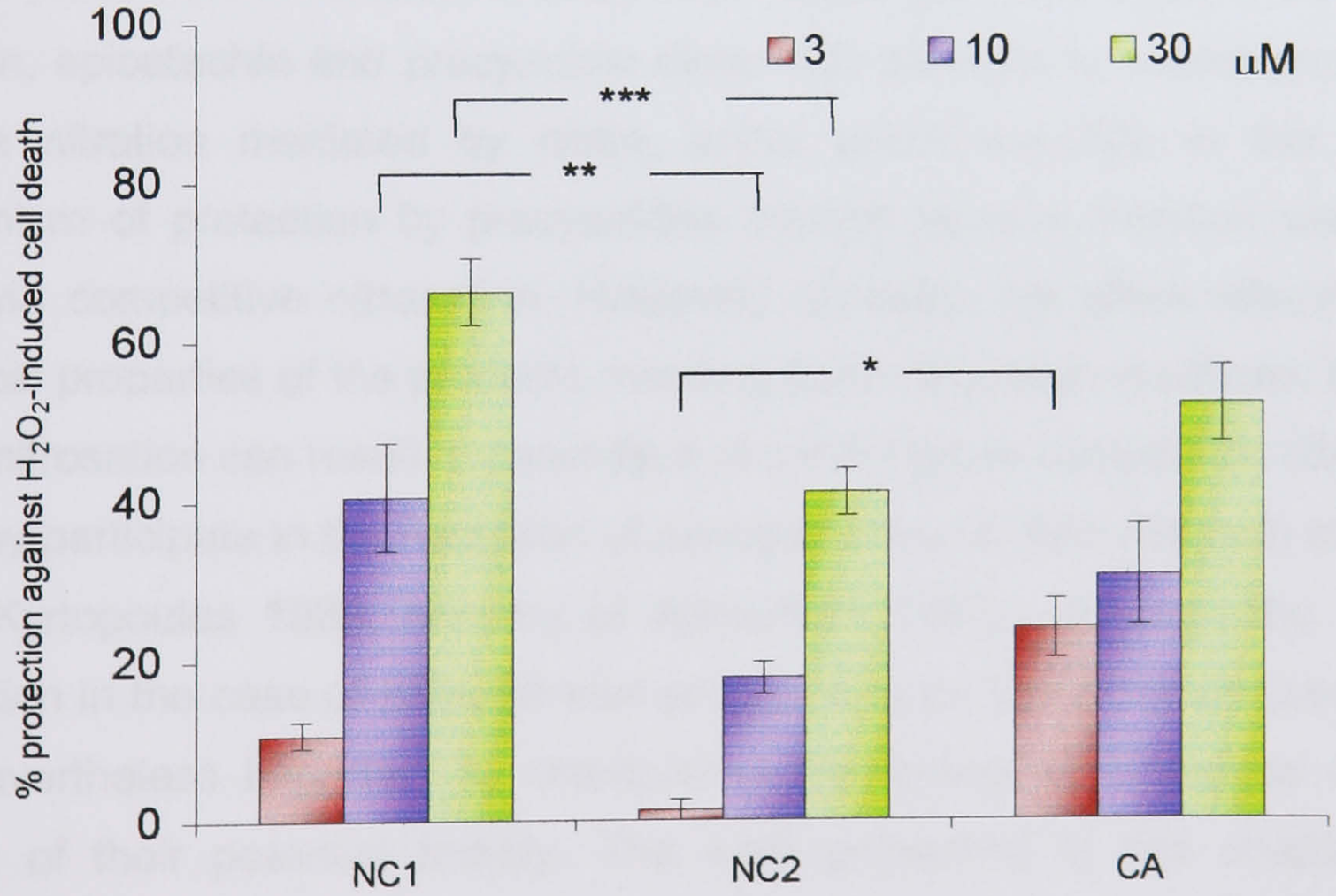


Fig 7.4 Effects of NCs and catechin on H_2O_2-induced loss of cellular function in fibroblasts. Data are presented as means ± SEM [n=3]. Treatments of NC1, NC2 and catechin at the same concentrations were compared with each other using one-way ANOVA with Tukey-Kramer multiple comparisons post *hoc* tests (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

7.3 DISCUSSION

Nitrate is present widely in green leafy vegetables and drinking water. Our diet contains a relatively large amount of nitrate, with a daily consumption of approximately 52 mg (Ysart *et al.,* 1999). Until recently, nitrate has been considered a harmful dietary component, associated with various diseases (McKnight *et al.,* 1999). Nitrate itself is in fact relatively harmless to human. It is the conversion of nitrate to nitrite by bacteria residing on the surface on the tongue that has been causing concern over many years. Nitrite is rapidly protonated to nitrous acid under the acidic environments of the stomach. Nitrous acid then decomposes spontaneously giving rise to the production of various RNS (Leaf *et al.,* 1989, McKnight *et al.,* 1997). One of the mechanisms whereby RNS contributes to the pathogenesis of diseases is via nitration of protein tyrosine residues. Enhanced levels of nitrotyrosine has been observed in the pathogenesis of many diseases, including Alzheimer's disease (Smith *et al.,* 1997), atherosclerosis (Beckman 1994), myocardial inflammation (Kooy *et al.,* 1997), lung cancer (Pignatelli *et al.,* 2001) and gastric cancer (Goto *et al.,* 1999).

Results presented in Chapter 3 show that monomeric and dimeric procyanidins (e.g. catechin, epicatechin and procyanidin dimer B2) are able to confer protection against tyrosine nitration mediated by nitrite, under conditions akin to the stomach. The mechanism of protection by procyanidins against tyrosine nitration was proposed to occur via competitive nitrosation. However, concerns are often raised regarding the biological properties of the products resulting from nitrosation reactions. It is well known that N-nitrosation can result in generation of carcinogenic compounds (Sen *et al.,* 1969) that may participate in the formation of cancers in the GI tract (Bartsch *et al.,* 1992, Bos 1989, Kyrtopoulos 1989, Ministry of Agriculture 1987). Although the mechanism of nitrosation in the case of catechin was proposed to be C-nitrosation (see Chapter 3), it was nevertheless important to characterise the biological properties of NCs, in the context of their potential toxicity. The work presented in this chapter focuses on examining the biological properties of NCs, with emphasis on their cytotoxicity and bioactivity.

Previous studies have reported contrasting observations regarding the mutagenic/carcinogenic properties of nitrosated flavonoids using the Ames/*Salmonella* mutagenicity test (Ames *et al.,* 1973). Genotoxic carcinogens can be detected by this

Biological properties of nitrosated catechins

assay based on the assumption that chemical substances with mutagenic properties are likely to be carcinogens. The test employs mutant histidine-dependent *Salmonella* strains that are unable to grow on a minimal media agar plate containing traces of histidine. Mutagens/carcinogens are identified by growth of the *Salmonella* strains through reversion of the mutation (Mortelmans and Zeiger 2000). Using the Ames assay (strain TA 100) the mutagenicity of twenty two flavonoids following incubation with nitrite in acid has been studied (Rueff *et al.,* 1995). Fifteen flavonoids tested positive in the Ames assay upon nitrosation, with quercetin and catechin showing the highest mutagenic activity amongst the flavonoids tested (Rueff *et al.,* 1995). By contrast, Tanaka *et al*., (Tanaka *et al.,* 1998) reported no mutagenicity in nitrosated products from tea extracts, containing catechin, using the same assay. Because of this limited and inconsistent information on the bioactivity of nitrosated flavonoids, it was difficult to predict the properties of NCs without further investigations. Several studies were therefore carried out to investigate the cellular activity of nitrosated catechins in this section.

Following production of NCs in the stomach, the first site to come into contact with these compounds is the small intestine. Initial studies were performed to study the potential toxicity of NCs using Caco-2 cells as a model of the intestine. Cells were cultured in the presence of test compounds for up to 21 days and their potential toxicity was evaluated by investigating their influence on cell proliferation. The toxicity of catechin on Caco-2 cells was studied as a comparison to NCs. At all three time points studied catechin lacked toxic potency towards Caco-2 cells, with the exception of 7 days treatment at 30 μM level. It is not clear why treatment with catechin at 30 μM for 14 and 21 days did not demonstrate any toxic effects. One possibility is that after 14 and 21 days, cells had recovered from the initial toxic effects induced during the previous 7 days treatment with 30 μM catechin. By contrast, no anti-proliferative effect was observed at any nitrosated catechins concentration compared with control cells after 7 days of treatment. Moreover, no significant difference was observed in cell proliferation between NCs and catechin, except at 30 μM, suggesting that nitrosation of catechin is unlikely to alter its function significantly. At 14 and 21 days supplementation, similar results were obtained for nitrosated catechins, with no anti-proliferative effect observed after treatment. These results imply that NCs and the native catechin did not influence the proliferation of Caco-2 cells if cultured in the presence of these

compounds. The results also provide evidence that nitrosation of catechin does not infer toxicity to the compounds, and thus are unlikely to be toxic to human intestinal cells following consumption of dietary components rich in these flavonoids, such as chocolate, green tea and red wine.

Despite previous studies having described the high mutagenicity with NCs using the Ames test (Rueff *et al.,* 1995), no adverse effect was observed here. In the Ames test the effects of chemical substances are evaluated using *Salmonella typhimurium*, a prokaryote, which is not an ideal model of the human body. The cell wall of prokaryotes is made up of peptidoglycan. Unlike human cell membranes, it is not a regulatory structure. In addition, the prokaryotic cell wall is porous and will allow passage of substances small enough to pass through (e.g. most antimicrobial agents). Nitrosated catechins are therefore likely to be taken up by *Salmonella typhimurium*, which is in contrast to their impermeability across Caco-2 monolayers (Chapter 5), and rat small intestine (Chapter 6). As such, the differences in the toxicity of nitrosated catechins observed in *Salmonella typhimurium* and in human cells probably arise from the difference in their absorption across the cell wall/membrane. Furthermore, in the Ames test a mixture of rat liver enzymes is normally included in the culture medium to compensate for the lack of mammalian metabolism. Based on the fact that the mutagenicity of many chemicals is a consequence of their metabolism, it is possible that NCs became mutagenic when they were exposed to the liver metabolising enzymes in the Ames test. By contrast, NCs are not metabolised by human cells, as demonstrated with the Caco-2 cells and the rat small intestine models (see Chapter 5&6). Thus the discrepancy in the observations made in the present study and those reported by Rueff *et al*., (1995) using the Ames test could also result in part from differences in membrane permeability and metabolism.

Procyanidins are receiving continue interest, owing to their beneficial activities towards human health. Accumulating evidence has demonstrated a potential role of procyanidins as protective agents against cardiovascular diseases (Karim *et al.,* 2000, Kondo *et al.,* 1996, Murphy *et al.,* 2003, Osakabe *et al.,* 2001, Rein *et al.,* 2000, Wan *et al.,* 2001, Waterhouse *et al.,* 1996), as neuroprotective agents (Inanami *et al.,* 1998, Schroeter *et al.,* 2001), and anti-cancer agents (Carnesecchi *et al.,* 2002, Romanczyk 1997). It has been reported that following 18h pre-treatment with epicatechin, human

fibroblasts (FEK4 cell line) were protected against oxidative-stress-induced cell death (Spencer *et al.,* 2001, Spencer *et al.,* 2001). In the present study, the same model was used to investigate whether NCs would confer similar protection to fibroblasts following exposure to H_2O_2. Supplementation of either NC1 or NC2 alone (for 18h) was not found to induce any toxic effects towards cell viability, with the exception of 10 μM NC1. The reason for this observed toxicity is unclear, since at higher concentrations (30 μM) no effects on cell viability were observed. In contrast, 30 μM catechin was found to reduce cell viability, contrary to the effects promoted by its epimer, epicatechin at a similar concentration (Spencer *et al.,* 2001). It was proposed in the same study that epicatechin confers protection against H_2O_2 via interactions with components of the cell signalling pathway (Spencer *et al.,* 2001). The disparity observed between toxicity of catechin and epicatechin could possibly arise from differences in their abilities to interact with signalling pathways, as epicatechin is better absorbed than catechin (Baba *et al.,* 2001).

In summary, nitrosated catechins were found to be non-toxic over the concentration range of 3 to 30 μM (apart from 10 μM NC1) in the human fibroblast model, suggesting that NCs are probably not harmful to human cells. However, it is difficult to make direct comparisons between results obtained from the Caco-2 and the fibroblast model. Cells were cultured in the presence of test compounds continuously in the former model, and the long-term toxicity of test compounds was evaluated by studying their influence on cell proliferation. By contrast, in the human fibroblast model cells were treated for a relatively short time (18h), and toxicity was evaluated through the ability of test compounds to trigger apoptotic cell death. Nevertheless, both models provide evidence to indicate that NCs, up to 30 μM, are not toxic to human cells.

The next biological property of NCs investigated, was their ability to protect against H_2O_2-induced cell death in fibroblasts. The physiological concentration of H_2O_2 varies at different sites of the body. Some studies have claimed substantial levels of H_2O_2 (up to ~35 μM) in human blood plasma (Lacy *et al.,* 1998, Varma and Devamanoharan 1991), but others have claimed levels to be very low, at or close to zero (Frei *et al.*, 1988). The latter data seem more credible, since H_2O_2 added to human plasma disappears rapidly, as a result of rapid interactions with ascorbate and heme proteins, but also degradation by catalase (Halliwell *et al.*, 2000). However, levels of H_2O_2 at or below about 20-50 μM

Biological properties of nitrosated catechins

seem to have limited cytotoxicity to many cell types. Earlier studies using sub-lethal doses of H_2O_2 (<150 μM) (Chen *et al.*, 2000) have reported the involvement of apoptotic processes in the cell damage of fibroblasts (Chen *et al.*, 2000, Spencer *et al.*, 2001). Apoptosis is a form of programmed cell death characterised by the morphological changes of nuclear condensation and cell shrinkage (Hale *et al.*, 1996). Epicatechin has been reported to protect against H_2O_2-induced cell death in fibroblasts and neurons at concentrations up to 30 μM (Spencer *et al.*, 2001, Spencer *et al.*, 2001). Using a similar concentration range to those aforementioned reports, nitrosated catechins and catechin were shown to afford similar protection against H_2O_2-induced cell death to fibroblasts. Compared to catechin, a similar degree of protection was observed when the cells were pre-treated with NC1 and NC2. This further substantiated previous observations that nitrosation of catechin does not result in significant alteration in its biological properties.

The mechanism by which NCs exert their cytoprotection *in vivo* was not addressed in the present study. Until recently the ability of flavonoids to protect against ROS and RNS has been attributed to their antioxidant properties. Although the Trolox equivalent antioxidant capacity (TEAC) values, which is designed for chemical assessment of the reducing properties of antioxidants through their hydrogen-donating abilities (Re *et al.*, 1999), for NCs have not been determined, nitrosation of catechins would probably influence their antioxidant potentials. Based upon the observation that no significant differences were observed between the protection elicited by NCs (apart from 3 μM NC2) and catechin against H_2O_2, it could be argued that their hydrogen-donating properties are not their primary mechanism of protection. Recently, there is growing evidence to suggest that flavonoids have mechanisms of action independent of their conventional antioxidant activities. One example is their ability to interact with cellular signalling pathways (Musonda and Chipman 1998, Tsai *et al.*, 1999), for instance with protein kinase C (PKC), which is a key player in many cellular responses including cell multiplication, apoptosis and transformation (Gamet-Payrastre *et al.*, 1999). Flavonoids have been shown to inhibit PKC activities, thereby preventing damage to cells (Bastianetto *et al.*, 2000, Gamet-Payrastre *et al.*, 1999). Furthermore, activation of the mitogen-activated protein kinase (MAPK) signalling cascades by quercetin (at low concentrations) has been reported, leading to expression of survival genes (c-Jun) and defensive genes (phase II detoxifying enzymes), resulting in survival and protective

Biological properties of nitrosated catechins

mechanisms (homeostasis response) (Kong *et al.,* 2000). In a recent study by Schroeter *et al.*, (Schroeter *et al.,* 2001) epicatechin was reported to protect primary striatal neurons from oxidative stress-induced toxicity via modulation of the c-Jun N-terminal Kinase (JNK) signal transduction pathway, since activation of JNK contributes to an apoptotic response. Other mechanisms reported in literature include alteration of enzymatic activity, such as inhibition of xanthine oxidase. Xanthine oxidase catalyses oxidation of both hypoxanthine and xanthine to uric acid, producing superoxide radical ($O_2^{\bullet-}$) and H_2O_2 as by-products. Through binding to the enzyme, procyanidins were reported to inhibit the enzyme's activity, thereby reducing the production of $O_2^{\bullet-}$ and H_2O_2 (Moini *et al.,* 2000). The flavonoid quercetin has also been shown to reduce H_2O_2-induced calcium dysregulation in cells, which has been implicated in various neurodegenerative diseases in aging (Wang and Joseph 1999). Furthermore, in a recent study by Spencer *et al.*, (Spencer *et al.,* 2001) epicatechin and its *in vivo* metabolite, 3′-O-methyl epicatechin were shown to protect human fibroblasts from oxidative stress-induced cell death involving a mechanism independent of their antioxidant properties. The level of protection elicited by 3′-O-methyl epicatechin was not significantly different from that of epicatechin, despite the latter being a much better antioxidant, as determined using the TEAC assay. The TEAC values of epicatechin and 3′-O-methyl epicatechin, relative to Trolox, were 4.8 and 1.8, respectively. Moreover, epicatechin and 3′-O-methyl epicatechin were shown to suppress caspase-3 activity (Spencer *et al.,* 2001). Caspase-3 was studied as a marker for apoptosis, since caspases are proteases that serve as primary drivers of apoptosis (Thorburn 2004). In view of this, it is possible that the mechanism of protection by catechin and nitrosated catechins against H_2O_2-induced damage in human fibroblasts involves (similar to epicatechin) interaction with cellular signalling pathways. Based upon this hypothesis, and the pharmacokinetic properties identified for NCs and catechin, there are two possible mechanisms by which such interactions with cell signalling pathways might occur:

1. Firstly nitrosated catechins and catechin promote their effects via different mechanisms. The ability of catechin to protect against H_2O_2 might occur via interactions with intracellular signalling mechanisms, such as suppression of caspase-3 and inhibition of JNK, similar to that promoted by its epimer, epicatechin. By contrast, NCs, which did not exhibit permeability across a Caco-2 monolayers

nor an *ex vivo* model of the small intestine, might have limited entry into the intracellular milieu (see Chapters 5 & 6). Therefore NCs could possibly exert their biological properties via interactions with extracellular receptors. An example of this is the agonist/antagonist activity of green tea extracts on the aryl hydrocarbon receptors, which mediates the transcriptional activation of CYP1A1 and CYP1A2 (Williams *et al.,* 2000). Evidence for the interactions of catechin with cellular receptors, such as the aryl hydrocarbon receptor (AhR) have been reported recently (Palermo *et al.,* 2003, Zhang *et al.,* 2003).

2. The second hypothesis is that both nitrosated catechins and catechin exert their biological properties by a similar mechanism, via interactions with extracellular receptors. This implies that the proposed (downstream) effects of catechin on caspase-3 and JNK are the result of interactions between catechin and extracellular receptors. The fact that catechin is absorbed into human cells may not be essential towards its biological activities.

Another interesting observation was that significantly higher level of protection by NC1 at 10 and 30 μM compared to NC2 was seen. It was not possible to delineate their difference in protection against oxidative stress at this stage, without further information on their structure. Further studies would be necessary to elucidate the precise structure of the nitrosated compounds, for example with nuclear magnetic resonance to identify the exact position of nitrosation. This would be useful for the understanding of their structures, and hence the difference in their effectiveness in protecting cells against oxidative stress. In summary, these findings suggest that NCs, produced from the inhibition of tyrosine nitration, are unlikely to exert toxic effects *in vivo*. Moreover, nitrosated catechins were able to offer protection against oxidative stress-induced cytotoxicity to human fibroblasts.

CHAPTER EIGHT

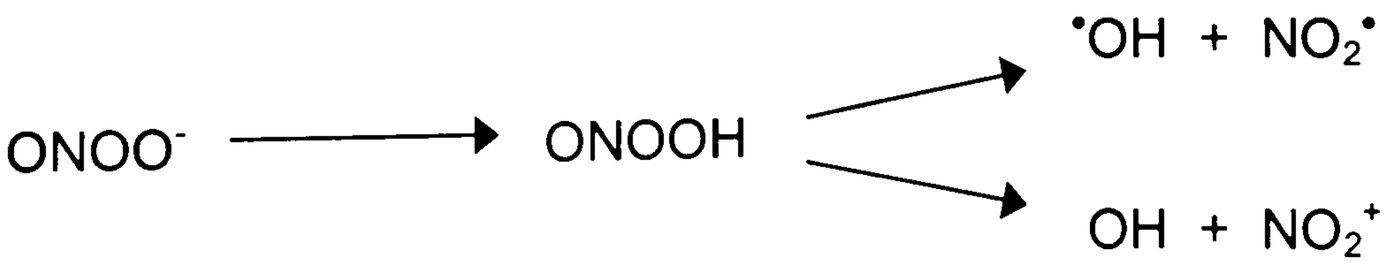
General Discussion

General discussion

Nitration of protein tyrosine residue is one of the mechanisms whereby reactive nitrogen species (RNS) contributes to the pathogenesis of many diseases. Recently, the ability of flavonoids to scavenge RNS has been demonstrated (Arteel and Sies 1999, Haenen and Bast 1999, Oldreive *et al.,* 1998, Pannala *et al.,* 1997, Pannala *et al.,* 1998), implicating their potential role in the protection against diseases involving the actions of RNS. The outcome of the studies presented in this thesis is that monomeric (and dimeric) procyanidins are capable of exerting protective effects against tyrosine nitration induced by acidic nitrite under conditions similar to the stomach. An IC_{50} of 51.9 μM was determined for the inhibition of 3-nitrotyrosine formations by catechin monomer. Epicatechin dimer was more effective than catechin in preventing acidic nitrite-mediated tyrosine nitration. At 50 μM, epicatechin dimer exhibited 66.9% inhibition of tyrosine nitration while catechin monomer only exhibited 48.6% inhibition. The mechanisms underlying the protection of procyanidins against acidic nitrite was investigated using LC-MS/MS for characterisation of the reaction products. Monomeric procyanidins undergo dinitrosation reaction while dimeric epicatechin undergo mono-nitrosation as well as dinitrosation.

It has been suggested that the intercepting of peroxynitrite-mediated tyrosine nitration by flavonoids with catechol structure in the B ring occurs via a reductive process, leading to oxidation of the catechol group (Kerry and Rice-Evans 1998, Pannala *et al.,* 1997). By contrast, flavonoid structures with monophenolic B rings are competitively nitrated (Pannala *et al.,* 1998). Thus the mechanism by which catechins, which contain the catechol structure, interacts with the RNS derived from nitrite in acid clearly contrasts with that from peroxynitrite.

The chemistry involved in the formation of RNS from peroxynitrite and nitrite in acid varies greatly. Peroxynitrite is a reactive and short-lived species produced from nitric oxide and superoxide via a radical-radical reaction *in vivo*. At physiological pH peroxynitrite protonates to form peroxynitrous acid, which decomposes rapidly to form a mixture of reactive products (Hughes 1968, Koppenol *et al.,* 1992), as depicted below:



Either homolytic fission can take place, which gives rise to nitrogen dioxide radical ($NO_2^{\bullet}$), or heterolytic fission resulting in production of nitronium ion (NO_2^+). For acidic nitrite, decomposition of nitrous acid is spontaneous, resulting in production of different RNS:

$NO_2^- + H^+ \longrightarrow HNO_2$ (p*K*a 3.2 – 3.4)

$HNO_2 + H^+ \longrightarrow H_2NO_2 \longrightarrow H_2O + NO^+$

$3HNO_2 \longrightarrow H_2O + 2NO^{\bullet} + NO_3^-$

$2HNO_2 \longrightarrow H_2O + N_2O_3$

$N_2O_3 \longrightarrow NO^{\bullet} + NO_2^{\bullet}$ (Leaf *et al.,* 1989, McKnight *et al.,* 1997)

One of the products in the decomposition of nitrous acid is dinitrogen trioxide (N_2O_3). Nitrous acid and dinitrogen trioxide are strong nitrosating species or NO^+ donors, which are capable of nitrosating nucleophiles such as thiols, amines and aromatics. Interactions with nitrosating species would result in the overall effect of $NO^{\bullet}$ addition i.e. nitrosation or nitrosylation (Schroeter *et al.,* 2002).

Other studies have shown that the reaction of nitric oxide ($NO^{\bullet}$) with a catechol moiety in the form of dopamine, under physiological pH, leads to a similar mechanistic sequence of generation of RNS. Dinitrogen trioxide can be produced via oxidation of $NO^{\bullet}$ when exposed to atmospheric O_2, as described below:

$2\ NO^{\bullet} + O_2 \longrightarrow 2\ NO_2^{\bullet}$ (Good 1997)

$NO^{\bullet} + {}^{\bullet}NO_2 \longrightarrow N_2O_3$ (Olanow and Tatton 1999)

However, upon interaction of dopamine with $NO^{\bullet}$, rather than undergoing nitrosation as the sole mechanism as in the case of procyanidins, dopamine was found to undergo nitrosation with subsequent oxidation during prolonged exposure to air (Rettori *et al.,* 2002). This is similar to tyrosine nitration, which has been proposed to occur via a two-step mechanism; nitrosation and subsequent oxidation (Knowles *et al.,* 1974) (Fig 8.1). The discrepancy between interactions of procyanidins and dopamine with RNS (or N_2O_3) was probably due to the difference in their stability. In contrast to the monophenolics dopamine and tyrosine, the 3-membered ring structure of procyanidins enable electron delocalisation and thus providing additional stability to the structures. It is likely that the enhanced stability of procyanidins

prevent further oxidation from taking place compared to the monophenolics. It was therefore hypothesised that nitration of polyphenols may proceed via a two-step mechanism similar to tyrosine nitration. In the case of catechin and procyanidins, the presence of the catechol moiety, possibly due to their reducing abilities, and their 3-membered ring structures prevents the reaction proceeding to the subsequent oxidation step. The overall result on interaction of catechin and procyanidins with acidic nitrite is therefore nitrosation.

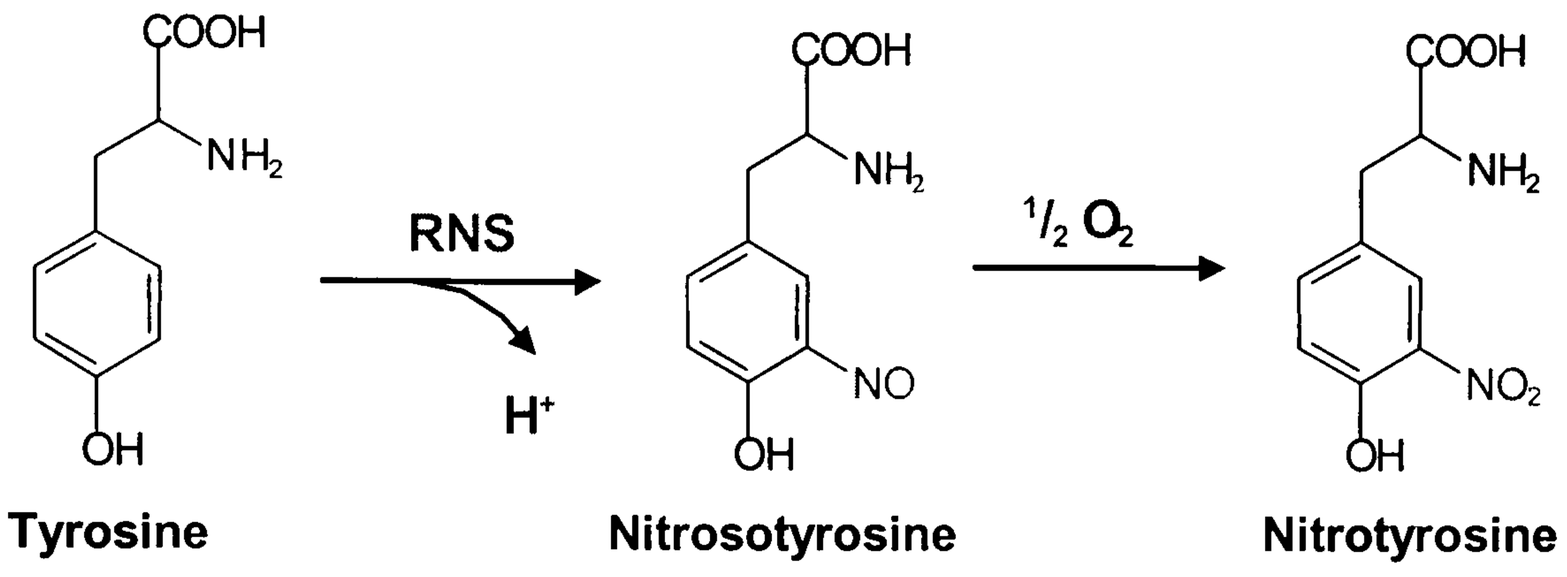
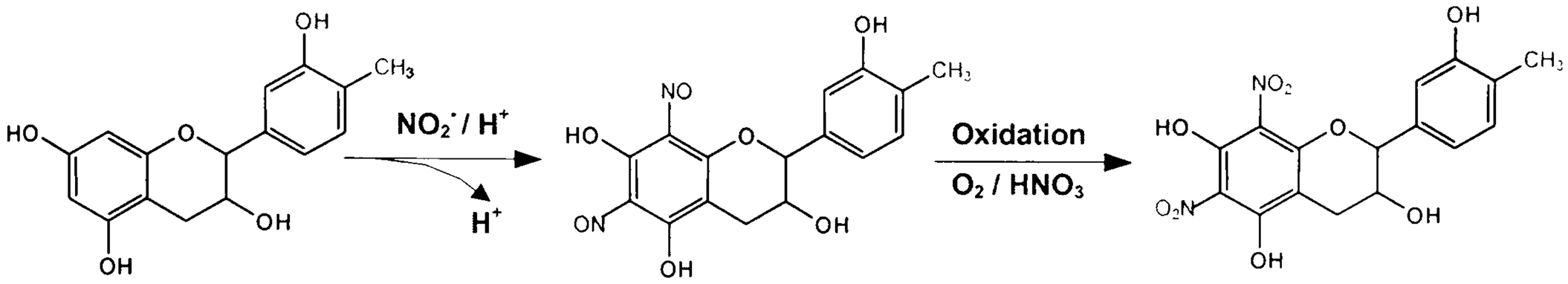


Fig 8.1 The mechanism of tyrosine nitration by RNS.

Based upon this hypothesis, further investigations were performed using flavonoids without the catechol structure, including hesperetin and methylated epicatechin, to elucidate the influence of the catechol structure on flavonoid reactivity with acidic nitrite-derived RNS. The major products obtained on interaction of hesperetin, which does not possess the catechol structure, with acidic nitrite were nitrated hesperetin. In addition, a small amount of nitrosated hesperetin was produced. This provides further evidence to strengthen the hypothesis that the chemistry of the catechol structure in the B ring is the major determinant of nitrosation by RNS derived from acidic nitrite. The presence of the catechol structure prevents reactions from proceeding to the subsequent oxidation step. However, on interaction with acidic nitrite, 3′ and 4′ methylated epicatechin behaved rather differently compared to hesperetin. The predominant mechanism was found to be, similar to epicatechin, nitrosation. The major difference observed between epicatechin and methylated epicatechin was the presence of nitro- and dinitro- products (as in the case of hesperetin) in the latter. The main difference between hesperetin and methylated epicatechin is that the major products obtained from hesperetin were nitrated whereas nitrosated products were mainly obtained from methylated epicatechin. It was therefore concluded that a contributory influence of the flavonoid C ring

structure on their reaction chemistry could not be ruled out. Nevertheless, the major determinant of the reaction profile of flavonoids with acidic nitrite derived-RNS is the presence of catechol structures in the B ring, as depicted in Fig 8.2.

A. Flavonoids without the catechol moiety, e.g. 3′ O-methyl epicatechin



B. Flavonoids with the catechol moiety, e.g. epicatechin

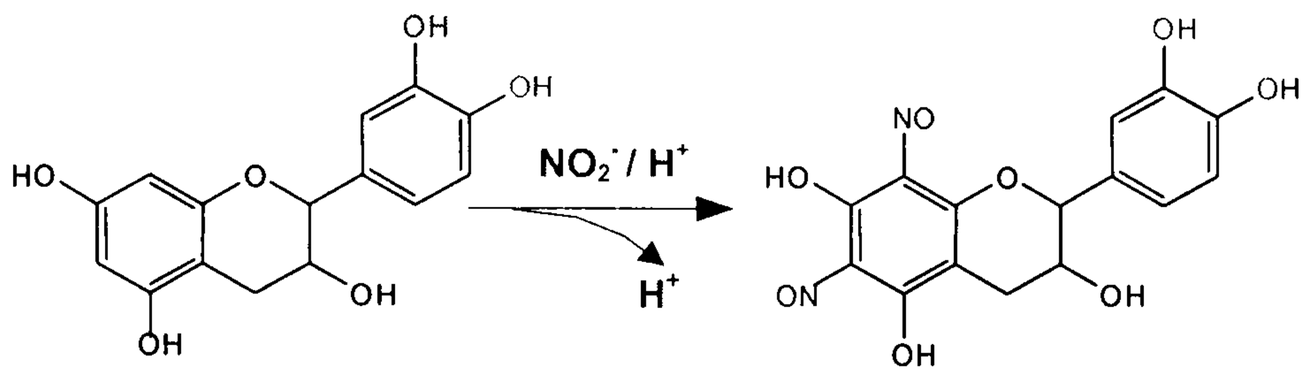


Fig 8.2 Proposed mechanisms of interaction between flavonoids and acidic nitrite. (A) Flavonoids without the catechol moiety are nitrated by acidic nitrite via a two-step mechanism; whereas (B) flavonoids with the catechol moiety are nitrosated.

Another major pubic health concern about high dietary nitrate is the potential formation of carcinogenic N-nitrosamines, from nitrite and secondary amines in the stomach (Sen *et al.,* 1969). N-nitrosamines are a large group of potent carcinogens that have been suggested as a major risk factor towards development of gastric cancer (Brown 1999, Goldman and Shields 2003, Hecht 1997). Inhibition of N-nitrosamine formation by antioxidants in fruit and vegetables, such as vitamin C (Hansson *et al.,* 1994, Tannenbaum *et al.,* 1991), has been proposed as an approach towards prevention of N-nitrosamine carcinogenesis. It is possible that dietary monomeric and dimeric procyanidins could provide similar protection against gastric cancer through their ability to scavenge RNS derived from nitrite in the stomach. Furthermore, *Helicobacter pylori* infection has recently been identified as a major cause of gastritis, which is a precursor of gastric cancer (Correa 1995, Forman 1998, Nomura *et al.,* 1991, Parsonnet *et al.,* 1991, Sipponen 1995).

Helicobacter pylori infection results in a chronic inflammatory response leading to production of ROS via recruitment and activation of monocytes and neutrophils (Sipponen 1995), and production of RNS via synthesis of inducible nitric oxide synthase (Nathan 1997, Nussler and Billiar 1993). Overproduction of ROS and RNS leads to oxidation, nitration and nitrosation of biomolecules, resulting in protein, DNA and tissue damage (Pignatelli *et al.,* 2001). Individuals eating 5-20 servings of fruit and 5-20 servings of vegetables each week have about half the risk of stomach caner, owing to the protection of antioxidants against ROS and RNS (Cummings and Bingham 1998). Consequently, the consumption of procyanidins can be beneficial to human health via their ability to inhibit tyrosine nitration, and thus preventing modification of protein and enzyme properties. Moreover, procyanidins may be protective against development of gastric cancer via inhibition of the formation of carcinogenic N-nitrosamines, and the scavenging of ROS and RNS during infection by *H. pylori*.

The next question arises as to whether the ability of monomeric/dimeric procyanidins to intercept tyrosine nitration in the gastric milieu by competitive nitrosation, might lead to the absorption of such nitrosated flavonoids, and whether nitrosation would alter the biological property of flavonoids. To further the investigations this work especially focused on the flavanol catechin. Absorption of catechin has been demonstrated previously, mainly in the forms of glucuronide-, sulphate-, sulphoglucuronide-conjugates of non-methylated or methylated catechin (Harada *et al.,* 1999). Studies were therefore undertaken to investigate the effect of nitrosation on the bioavailability of catechin. As nitrosation of catechin was proposed to occur under the acidic environment of the stomach, the small intestine would be the first site of contact with these compounds. The Caco-2 permeability model was chosen as a suitable model to represent the human small intestine. Caco-2 cells were derived from a colonic adenocarcinoma (Fogh 1977), but they differentiate to resemble normal, differentiated enterocytes when cultured for 21 days (Artursson 1990, Hidalgo *et al.,* 1989, Pinto 1983). In the field of flavonoid research the Caco-2 model has been used to examine and demonstrate the permeability of several flavonoids, for instance quercetin (Walgren *et al.,* 1998), chrysin (Walle *et al.,* 1999), and epicatechin (Vaidyanathan and Walle 2001).

Permeability of 3-nitrotyrosine (P_{app} of 0.41 $\pm$ 0.05 x 10^{-6} cm/sec), determined using the Caco-2 model suggested its absorption by the small intestine after production in the stomach was likely to occur. Evidence for the incorporation of 3-nitrotyrosine into

proteins is limited, with α-tubulin being the only example obtained so far (Eiserich *et al.,* 1999). The replacement of tyrosine residues by nitrotyrosine induces a change in polarity that may alter protein/enzyme activity. Implication of tyrosine nitration *in vivo* includes alteration of cell morphology (Eiserich *et al.,* 1999), reduced antioxidant defence (MacMillan-Crow and Thompson 1999), impaired cellular function (Rohn *et al.,* 1999) and interference with the signalling cascade (Crow *et al.,* 1997, Gow *et al.,* 1996). By inhibiting the formation of 3-nitrotyrosine and their subsequent potential to incorporate into proteins, monomeric and dimeric procyanidins may provide important protection against the consequence of tyrosine nitration.

The Caco-2 model demonstrated permeability of catechin across the monolayer. In contrast, no evidence for transport of nitrosated catechins was obtained. Further information on the permeability of NCs was obtained using the isolated rat small intestinal model. Consistent with the results obtained in the Caco-2 model, no absorption was detected across the small intestine following 80 min perfusion with NCs. With regard to catechin, absorption was demonstrated across the rat small intestine. No catechin aglycone was detected in the serosal samples, but several peaks with catechin-like UV spectra were obtained. The amount of catechin absorbed across the jejunum was found to be 0.36% of the total perfused catechin. Previous observations made following perfusion with epicatechin have shown the formation of both glucuronide and O-methyl glucuronide conjugates (Kuhnle *et al.,* 2000). Based upon these findings and the effect of β-glucuronidase treatment observed in the current study, the metabolites were suggested to be a glucuronide-, an O-methyl-glucuronide- and an O-methyl conjugate of catechin according to their sequence of elution. One possible explanation for the lack of permeability of nitrosated catechins is that, catechin was detected in the serosal fluid mainly as glucuronide conjugates, which are usually attached to the 5 or 7 positions on the A ring of their structures. Since nitrosation of catechin was proposed to occur at the 6 or 8 positions on catechin, it is possible that addition of the nitroso groups result in steric hindrance, preventing glucuronidation of nitroso catechin. However, this does not explain the factors that hinder the initial uptake of nitrosated catechins, unless glucuronidation is a cellular membrane event and a pre-requisite for uptake of flavonoids. Another possibility could have been that interaction of NCs with efflux transporters was responsible for their efflux back into the small intestine, which would otherwise have been prevented if glucuronidation had taken place. On the other hand, studies undertaken in Chapter 5 (comparison of apical to basolateral

with basolateral and apical P_{app}s) suggested NCs are unlikely to be substrates of efflux pumps, therefore the second proposal seems unlikely.

Passive diffusion across the intestinal mucosa is the most common method of absorption, in which the compound has to diffuse across a series of barriers (Jackson 1987). The most restrictive barrier to absorption is the single layer of epithelial cells lining the mucosal surface of the small intestine. Permeability of compounds across the epithelial layer is in turn governed by numerous factors, including compound lipophilicity, solubility, metabolism by enzymes in the intestinal lumen, interaction with active transporters upon absorption as well as interactions with efflux systems. Nevertheless, a common approach to predicting absorption in the small intestine is based upon the lipophilicity of the compounds of interest (Artursson 1991). A positive relationship (R^2 = 0.58) between the P_{app}s of 20 drugs, with different structural properties and their lipophilicity has been demonstrated previously (Artursson 1991). Nitrosated catechins (cLog P = 0.92) are less polar than catechin (cLog P = 1.18), as such their absorption was predicted to be a likely event compared to catechin. Contrary to expectations, results obtained so far suggest that while nitrosated catechins are unlikely to be absorbed by the small intestine, catechin was able to permeate across the Caco-2 monolayers and isolated rat small intestine. When the permeability of different flavonoids was investigated and compared to their respective cLog P in the present study, it became apparent that only a weak correlation existed (R^2 = 0.42). This indicates that other mechanisms of transport, such as involvement of active and/or efflux transporters are likely to be involved in the uptake of flavonoids across the small intestine. Information on the involvement of active transporters in the permeability of flavonoids across the small intestine is scarce. Existing information is only available for flavonoid glycosides, with evidence demonstrating uptake of quercetin glucosides via the sodium-dependent glucose transporter (SGLT1) in Caco-2 cells (Walgren *et al.*, 2000) and isolated rat small intestine (Day *et al.*, 2003, Wolffram *et al.*, 2002). Little information is available in the literature with regard to the interaction of flavonoids and possible uptake by other transporters. A rapid, energy-dependent transport system was suggested to be present in mammalian aortic endothelial cells for the uptake of morin, as shown by the several-fold increase in morin uptake when ATP was added to the culture medium (Schramm *et al.*, 1999). The authors further suggested that the transport system in these cells uses hydroxylated phenolic compounds as substrates. However, it is not clear whether such transporters, or other active transporters capable of transporting flavonoids are present in

enterocytes forming the small intestine. If flavonoids can be transported via this mechanism, the addition of nitroso groups to catechin may influence the way it interacts with the transport system, thereby affecting their uptake into cells. Further studies are required to elucidate the mechanisms involved in the transfer of hesperetin, catechin and NCs across the small intestine.

While little is known about the involvement of active transporters during uptake of flavonoids, interaction of flavonoids with efflux transporters has received much more attention over the recent years. The small intestine expresses many efflux transporters on the mucosal surface, namely P-glycoprotein (P-gp) and the multidrug resistance protein (MRP) family, with the exception of MRP6 (Gottesman *et al.,* 2002). Interactions between flavonoids and MRP2, including quercetin, epicatechin, epicatechin gallate and chrysin have been reported to be associated with their efflux in Caco-2 cells (Vaidyanathan and Walle 2003, Vaidyanathan and Walle 2001, Walgren *et al.,* 1998, Walle 2003, Walle *et al.,* 1999). More information is available for the interaction of flavonoids with P-gp using different cell models. Hesperetin, naringenin, quercetin, epicatechin and its O-methylated metabolites have recently been reported to inhibit P-gp efflux of colchicine in Madin-Darby Canine Kidney cells transfected with the human MDR gene (MDCK-MDR1) (Youdim *et al.,* unpublished). Other studies have described the inhibitory effect of hesperetin on vincristine efflux from cultured mouse brain endothelial cells (Mitsunaga *et al.,* 2000), and quercetin on P-gp efflux of rhodamine 123 (R123) in rat hepatocytes (Chieli *et al.,* 1995). As P-gp is particularly abundant on the apical surface of enterocytes, interaction of catechin, epicatechin, hesperetin and quercetin with P-gp was studied by investigating their ability to inhibit R123 efflux, using MDCK-MDR1 cells. Apart from catechin, all three flavonoids were able to interact with P-gp. It was further suggested that the mechanism of inhibition by epicatechin, hesperetin and quercetin was via competitive inhibition with substrates of P-gp. Interestingly, apart from being the only flavonoid unable to interact with P-gp, no efflux of catechin was detected when it was loaded on the basolateral side. This further supports the hypothesis that inhibition of P-gp activity by flavonoids occur via a mechanism of competitive inhibition, with catechin not being a substrate and thus unable to interact with P-gp.

Studies described so far demonstrate the ability of monomeric and dimeric procyanidins to scavenge RNS produced in the gastric milieu. This would result in protection against tyrosine nitration, and possibly the subsequent implications in

various diseases. The final section of this thesis focuses on the biological activities of NCs in cell culture models. The absorption and metabolism of procyanidins, including interactions with efflux transporters present in cells, would significantly affect their subsequent bioactivity. The precise biological properties of procyanidins *in vivo* will depend on the extent to which they are absorbed and metabolised during transfer across the small intestine, in the liver and in the colon (Donovan *et al.,* 2001, Rice-Evans *et al.,* 2000, Rice-Evans 2001, Spencer 2003). Nitrosated catechins were demonstrated to be impermeable across the small intestine in the current study, suggesting the biological effects of these compounds are likely to be limited to the GI tract. The potential carcinogenic effects of nitrate and nitrite have attracted considerable research owing to their ability to react with secondary and tertiary amines, resulting in the production of carcinogenic nitrosamines (Sen *et al.,* 1969). While N-nitrosation and the related health consequence has been extensively studied and is now well understood, aromatic C-nitrosation has received much less attention. Previous studies have attempted to determine the mutagenicity of nitrosated flavonoids using the Ames assay. Contrasting results were yielded with one study demonstrating mutagenic effect of catechin upon nitrosation (Rueff *et al.,* 1995), while another study reported no mutagenicity in nitrosated products from tea extract (Tanaka *et al.,* 1998).

The toxicity of nitrosated catechins was investigated using two human cell models: the Caco-2 cells as a model of the intestine, and the FEK4 cells. When Caco-2 cells were cultured in the presence of NCs, no significant effect was observed in the proliferation of cells in general. Similarly, both nitrosated catechins and catechin did not display any toxic effects to the human fibroblasts following 18h treatment, nor did nitrosation of catechin affect their ability to inhibit H_2O_2-induced cytotoxicity. Furthermore, all three compounds were found to offer protection against H_2O_2-induced cytotoxicity to fibroblasts, with no significant difference observed in the degree of protection between NC1, NC2 and catechin. Apart from the antioxidant property of procyanidins, studies have demonstrated other biological/molecular properties. These include alteration of enzymatic activity, such as inhibition of xanthine oxidase, resulting in inhibition of $O_2^{\bullet-}$ and H_2O_2 production (Moini *et al.,* 2000), and interaction with signalling mechanisms in cells. Epicatechin has been reported to protect primary striatal neurons from oxidative stress-induced toxicity via modulation of the c-Jun N-terminal Kinase (JNK) signal transduction pathway, since activation of JNK contributes to an apoptotic response (Schroeter *et al.,* 2001). Furthermore, in a recent study by Spencer *et al.*, (Spencer *et al.,* 2001) epicatechin

and its *in vivo* metabolite, 3′-O-methyl epicatechin were shown to protect human fibroblasts from oxidative stress-induced cell death involving a mechanism independent of their antioxidant properties. Epicatechin and 3′-O-methyl epicatechin were shown to suppress caspase-3 activity, a marker for apoptosis (Spencer *et al.,* 2001). Based on this study two possible mechanisms by which NCs and catechin exert their protection against H_2O_2, via interactions with cellular signalling pathways, were proposed. It might be that catechin inhibits apoptosis via suppression of intracellular signals such as caspase-3, while NCs exert their biological properties via interactions with extracellular receptors, owing to their limited entry into cells. Alternatively, catechin and nitrosated catechins both offer protection via a common mechanism of interactions with extracellular receptors. In view of the similar degree of protection offered by catechin and nitrosated catechins, the second hypothesis was concluded to be more feasible. With regard to the different extent of protection demonstrated by NC1 and NC2, further information on the position of nitrosation is required to delineate the difference.

In considering the biological importance of dietary antioxidant, attention has usually been focussed on those that are absorbed through the GI tract, and the subsequent local effects of the metabolites. The work presented here demonstrates that flavonoids can offer significant protection to the stomach, as potent scavengers of acidic nitrite-generated RNS, resulting in inhibition of tyrosine nitration. With respect to the procyanidins, the mechanism of their protection against tyrosine nitration involves either mono- or dinitrosation. Through their ability to inhibit tyrosine nitration, and to scavenge RNS possibly resulting in prevention of N-nitrosamines formation, procyanidins could potentially protect against development of gastric cancer. However, the potential health benefits of procyanidins are not limited to inhibition of tyrosine nitration. Procyanidins can also prevent modification of other biomolecules, in particular DNA, owing to their ability to scavenge RNS (Halliwell *et al.,* 2001). Nitrous acid can deaminate DNA bases, producing mutagenic lesions. The major reactions included conversion of adenine to hypoxanthine, and of guanine to xanthine and oxanine (Zhao *et al.,* 2001). Although the potential protection of procyanidins on DNA modification was not investigated in the current study, it will nevertheless be an interesting subject for future investigations. Apart from nitrate and nitrite, the GI tract is exposed to a wide range of pro-oxidants to a degree unprecedented in other body tissues (Halliwell *et al.,* 2001). For instance, ingested food frequently contains iron ions, which may lead to generation of hydroxyl radical ($OH^{\bullet}$) by Fenton chemistry, and target the stomach, duodenum and

upper small intestine (Halliwell *et al.,* 2001). The ability of procyanidins to chelate iron (Morel *et al.,* 1993) could offer protection against free radical formation and the propagation of free radical reactions in the GI tract. Diets high in meat and fat have been linked to high rates of colorectal cancer by epidemiology studies. The association is usually attributed to the formation of N-nitroso compounds, derived from protein metabolism of meat and RNS, in the colon (Bingham 2000). N-nitrosation in the large intestine is positively influenced by dietary nitrate, and has been shown in animal models to be dependent on the presence of a gut flora (Massey *et al.,* 1988). High intake of fruit and vegetables is an established factor that reduces colorectal cancer risk (Cummings and Bingham 1998), possibly owing to the ability of antioxidants such as procyanidins to scavenge RNS, and inhibit formation of N-nitroso compounds. The amount of flavonoids absorbed by the body varies according to their subclasses, but the highest percentage absorption was estimated to be 52% for flavanols, suggesting considerable amounts of flavonoids would reach the intestine. The potential role of procyanidins in protecting the GI tract from oxidative damage is summarised in Fig 8.3.

Stomach (high concentration of flavonoids) • RNS scavenging – inhibition of tyrosine nitration – inhibition of N-nitrosation – inhibition of DNA modification • Iron chelating – inhibition of free radical generation
Small intestine (absorption of some flavonoids) • Iron chelating – inhibition of free radical generation
Colon/Rectum (considerable amount of flavonoids) • RNS scavenging – inhibition of N-nitrosation • Metal chelating – inhibition of free radical generation

Fig. 8.3 The potential role of procyanidins in protecting the GI tract from oxidative damage.

In conclusion, monomeric procyanidins is a good inhibitor of tyrosine nitration via a novel mechanism of nitrosation, which is likely to protect proteins and enzymes in the GI tract against nitration and nitrosation. Nitrosated catechins appear not to be absorbed by the small intestine, and were suggested to be unable to elicit any

biological effects beyond the GI tract. Most importantly, the end products of this reaction (nitrosated catechins) were found to be non-toxic to cultured cells, suggesting they are unlikely to pose significant health risk to human.

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APPENDIX

Appendix 1

Recipes for chromogenic test reagents

1. Dragendorff's reagent

- Stock solutions: mix bismuth subnitrate (oxynitrate; 1.7 g) with water (80 ml) and glacial acetic acid (20 ml). Add potassium iodide solution (50% w/v, 100 ml). Shake or stir until dissolved. Solution keeps indefinitely when stored in a dark bottle.
- Working solution: mix the stock solution (100 ml) with glacial acetic acid (200 ml) and make up to volume (1 litre) with distilled water. Keeps for 2-5 months when stored in a dark bottle.

2. Anisaldehyde spray

- Working solution: mix *p*-anisaldehyde (0.5 ml), glacial acetic acid (10 ml), methanol (85 ml and concentrated sulphuric acid (5 ml).

3. Naturstoff reagent + Polyethylene glycol 4000

- Naturstoff reagent (a 1% solution of diphenlboric acid-ethanolamine complex in methanol): mix 0.5 g DBAE in 50 ml MeOH
- PEG 4000 solution: mix 1g PEG 4000 in 20 ml ethanol

Appendix 2

Composition of Hank's Balanced Salt Solution for Caco-2 permeability studies

$CaCl_2 \cdot 2H_2O$	0.185 g/L
KCl	0.40 g/L
KH_2PO_4	0.06 g/L
$MgCl_2 \cdot 6H_2O$	0.10 g/L
$MgSO_4 \cdot 7H_2O$	0.10 g/L
NaCl	8.00 g/L
$NaHCO_3$	0.35 g/L
Na_2HPO_4	0.048 g/L
D-glucose	1.00 g/L

Appendix 3

P_{app} calculations for flavonoids

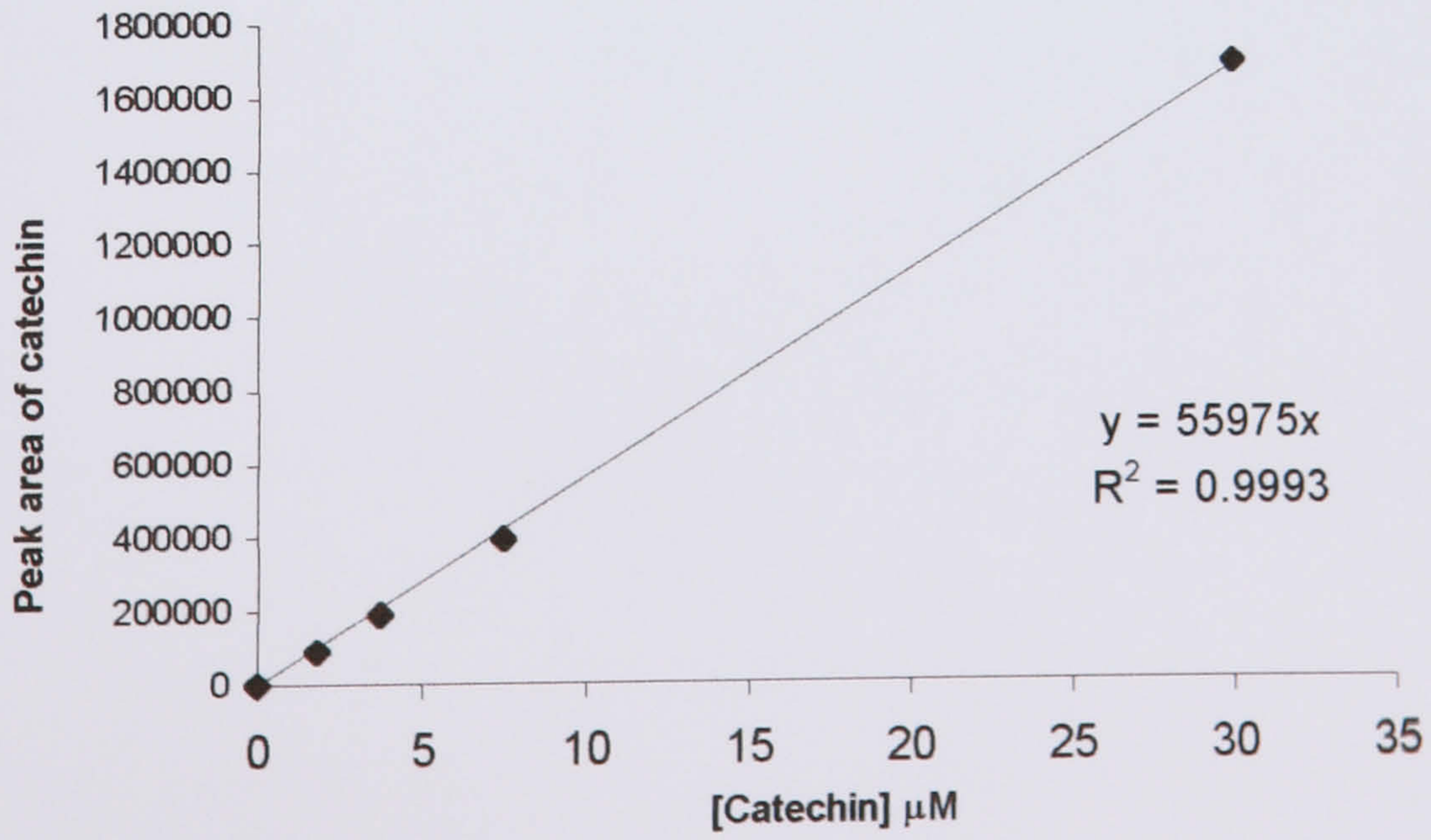
1) Working example for calculating A to B permeability (P_{app}) of 25 μM catechin

An aliquot of the transport buffer containing catechin was analysed by HPLC to accurately determine the concentration/amount of catechin present at the beginning of the experiments. Following permeability experiments, samples from both sides of the cell monolayers were collected at the end of experiments and analysed by HPLC for the presence of flavonoids. An example of the results is shown in the following table, with the peak areas of catechin obtained from HPLC illustrated in the 2nd column.

	Peak area	*Concentration (μM)*	*Total amount (μg)*
Time 0	1378456	24.62	3.57
Donor well	1290348	23.05	3.35
Receiver well	10113	0.40	0.12

Table 1. Representative results from the Caco-2 permeability experiment for determination of the A to B P_{app} of 25 μM catechin.

The concentrations of catechin present at the beginning of experiments, in the donor and receiver wells after 60 min incubation were determined using a standard graph generated from known fixed concentrations (μM) of catechin, as shown in Fig ??.



Standard graph of catechin.

P_{app} of catechin was calculated in mass terms, with the advantage that mass balance can be readily followed. The equation used is as follows:

$$P_{app} = V_D / (A.M_D) \times (\Delta M_R / \Delta t)$$

Where V_D is the volume of buffer in the donor compartment (cm^3), *A* is the surface area of the membrane (cm^2), $\Delta M_R / \Delta t$ is the amount of compound transferred to the receiver compartment over time (sec).

Total amount of catechin present was calculated based on the volume of buffer present in the donor and receiver wells were 0.5 and 1.0 ml, respectively. Using the above equation, P_{app} of 25 μM catechin was calculated, which was 0.45×10^{-6} cm/sec.

P_{app} of catechin as also calculated using another equation, which corrects for any compound retained in the monolayer:

$$P_{app} = V_D / (A.M_D - M_{cells}) \times (\Delta M_R / \Delta t)$$

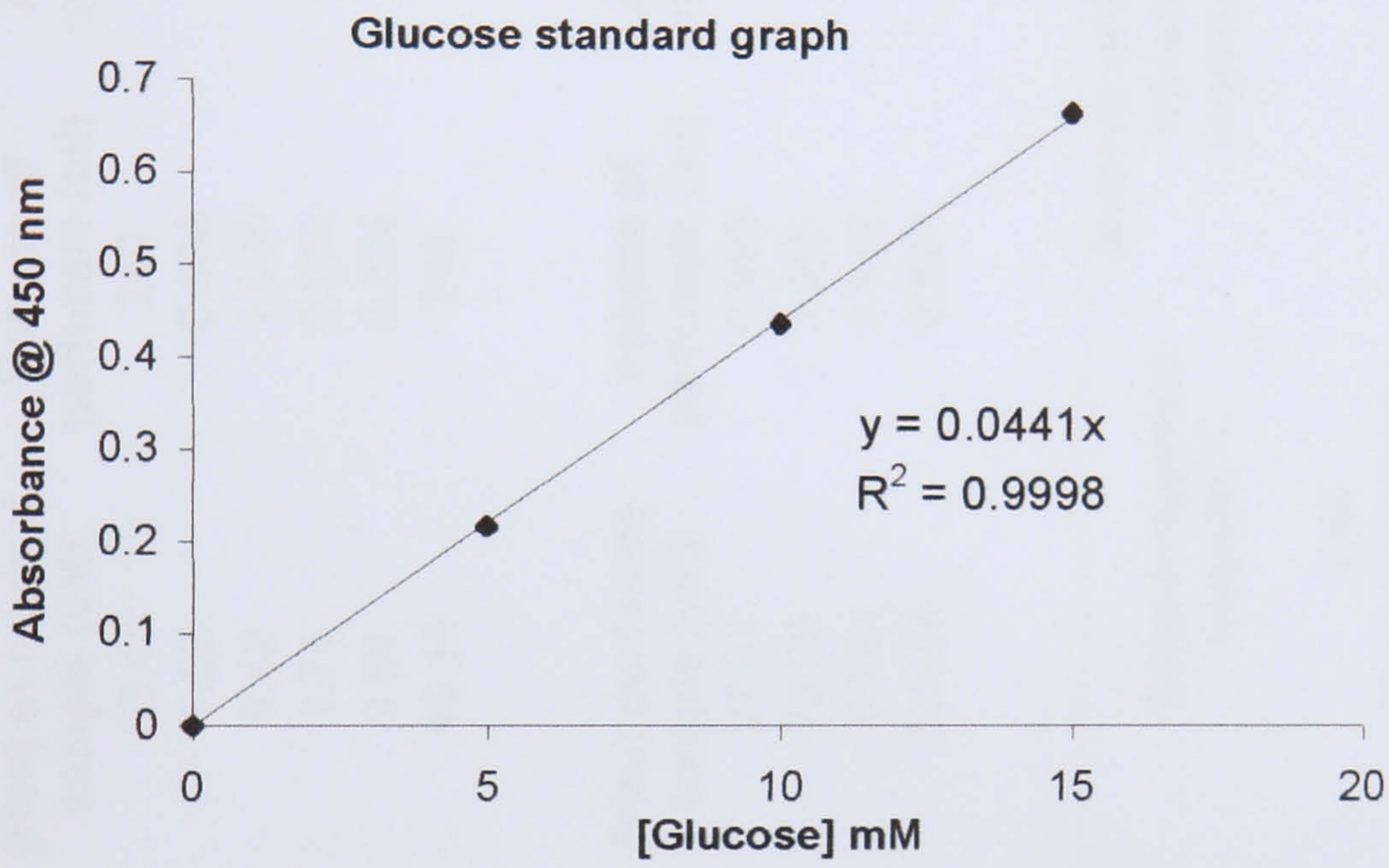
where M_{cells} is the amount of compound retained by the membrane.

Using this equation a P_{app} of 0.47×10^{-6} cm/sec was obtained.

Appendix 4

Calculation for absorption of flavonoids in small intestine

The viability of the isolated jejunum during the course of the experiment was determined by measuring glucose uptake, using the glucose oxidase assay. The standard graph of glucose used for the measurement is shown below:



Standard graph of glucose obtained using the glucose oxidase colourimetric assay.

Absorption of flavonoids across the rat jejunum was calculated as the amount of compound (ng) transferred to the serosal solution per cm of tissue per min. An example is shown in the table below:

1st experiment (7) **Length of jejunum (cm)** **32**

Sample	Dilution Factor	Hesperetin Peak area	[Hesperetin] (uM)	[Hes] in original sample (uM)	Volume of perfusate (ml)	Amount of Hes in sample (nmol)	Amount of Hes sample (mg)
Perfusion buffer	0.095	1216365	6.21	65.45	240	15708.95	4748.82
20 min	0.095	29135	0.15	1.58	0.938	1.47	0.45
40 min	0.095	7800	0.04	0.42	1.095	0.46	0.14
60 min	0.095	32408	0.17	1.74	0.932	1.63	0.49
80 min	0.048	33790	0.17	3.64	0.824	2.99	0.91
Waste buffer	0.095	913518	4.67	49.16	240	11797.78	3566.47

After B-glucuronidase treatment

Sample	Dilution Factor	Hesperetin Peak area	[Hesperetin] (uM)	[Hes] perfusate sample (uM)	Volume of perfusate (ml)	Amount of Hes in sample (nmol)	Amount of Hes sample (mg)
20 min	0.086	457791	2.34	27.37	0.938	25.67	7.76
40 min	0.043	663040	3.39	79.29	1.095	86.80	26.24
60 min	0.043	1420036	7.26	169.81	0.932	158.31	47.86
80 min	0.043	1953003	9.98	233.54	0.824	192.41	58.17

Amount of Hes sample (μg)	Amount of gluc. (μg)	Total hesperetin transfer %	Amount of Hesperetin transferred (ng/cm/min)
4748.82			
0.45	7.32	2.95	
0.14	26.10	**% as hesperetin**	54.70
0.49	47.37		
0.91	57.26	1.41	
3566.47		**% as glucuronide**	
Total absorbed	**Total absorbed**	98.59	
1.98	138.05		

Appendix 5

Sulphorhodamine B standard Graphs

The following figures depict the standard graphs of sulphorhodamine B absorbance (total biomass) vs. number of Caco-2 cells analysed using the sulforhodamine B assay. As shown in Figure A, the relationship between total biomass and cell number is linear from 0 to 240,000 cells per well, consistent with previous report (Carnésecchi *et al*, 2002). The original standard graph with 0 to 480,000 cells/well is shown in Figure B.

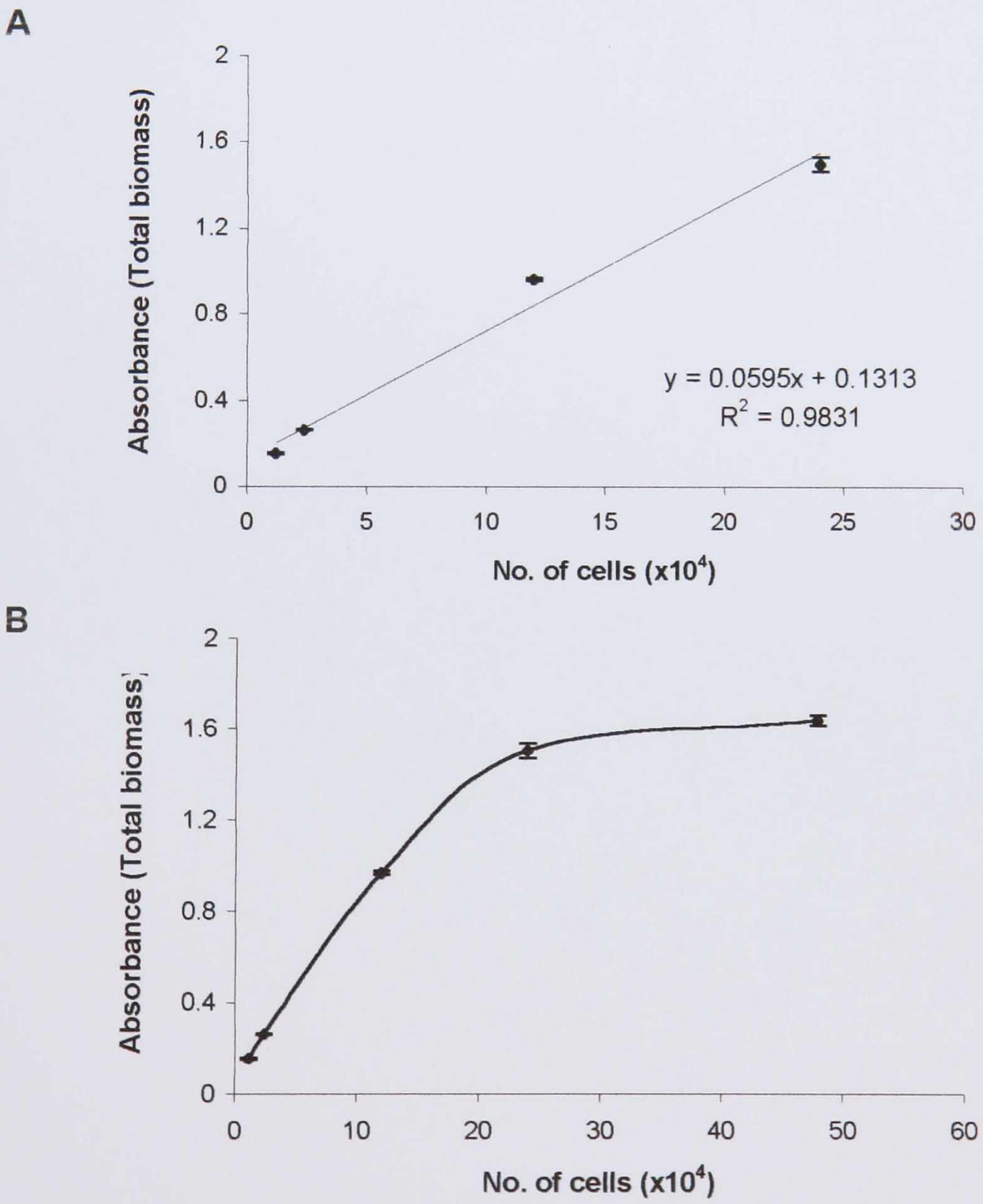


Figure 4. The correlation between absorbance (total biomass) and number of Caco-2 cells measured by the sulforhodamine assay: (A) 0 to 240,000 cells per well, (B) 0 to 480,000 cells per well.